

Research Paper

Regulatory effects of *Lactobacillus plantarum*-GMNL6 on human skin health by improving skin microbiome

Wan-Hua Tsai¹, Chia-Hsuan Chou¹, Ying-Ju Chiang¹, Ching-Gong Lin², Che-Hsin Lee^{3,4,5,✉}

1. Research and Development Department, GenMont Biotech Incorporation, Tainan, Taiwan
2. Bachelor Program in Cosmeceutical and Biotech industry, Department of Cosmetic Science, Chia Nan University of Pharmacy & Science
3. Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan
4. Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan
5. Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan

✉ Corresponding author: Dr. Che-Hsin Lee, Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan, 70 Lienhai Rd. Kaohsiung 80424, Taiwan. E-mail: chlee@mail.nsysu.edu.tw

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Received: 2020.08.05; Accepted: 2020.12.19; Published: 2021.01.01

Abstract

Bacteria response to their environment by producing some compounds which are used in cosmetic and pharmaceutical applications. Some probiotics can regulate immune response and modulate the symptoms of several diseases. Bacteria affect skin response to skin care products. Bacteria are thought to play an important role in acne incidence, skin moisture, and nutrient metabolism, but only a few studies have focused on the extracts of *Lactobacillus plantarum* in skin care. In this study, we identified that *L. plantarum*-GMNL6 enhanced collagen synthesis and the gene expression of serine palmitoyltransferase small subunit A. Meanwhile, *L. plantarum*-GMNL6 reduced the melanin synthesis, the biofilm of *Staphylococcus aureus*, and the proliferation of *Cutibacterium acnes*. Information from clinical observation during the ointment for external face use in people displayed that the syndromes of skin moisture, skin color, spots, wrinkles, UV spots, and porphyrins were improved. The diversification of human skin microbiomes was affected by smearing the face of volunteers with *L. plantarum*-GMNL6. Understanding the potential mechanisms of the action of *L. plantarum*-GMNL6 in dermatologic conditions promotes the development of care products.

Key words: *Lactobacillus plantarum*-GMNL6; probiotics; skin care; care products

Introduction

Some products of bacteria with anti-oxidative activity have been show to play an important role in skin care. Bacteria can produce some compounds in response to environmental stress. These compounds of bacteria are generally used in medicine, cosmetics, even in sports [1]. Some probiotics have immunomodulatory activities and modulate the symptoms of several diseases [2]. Previously, the extracts of *Rhodobacter sphaeroides* inhibited inducible nitric-oxide synthase expression in activated macrophages, and reduced inflammation in colitis model [3]. The probiotics are complementary and alternative medicines and is used for promoting health [4]. Some specific probiotic strains, including *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Bacillus*, and *Lactobacillus*, have been demonstrated for health

benefits [5]. Recently, *Lactobacillus* had been reported to ameliorate symptoms of diabetes in human studies [6]. *Lactobacillus* also reduced neuropsychiatric disorders, regulated blood pressure, provided physiological benefits, and exerted anti-inflammatory effects [7]. The pigmentary disorders and melanoma are abnormal melanogenesis is a feature of many human skin diseases. The wrinkles, melasma and age spots are often observed in skin lighterers in European. In Asia, the care products are used to make skin whiter and moisture [3]. Some probiotics is able to serve as a potential care products. Probiotics such as lactobacillus have been demonstrated to modulator skin symptoms [8]. *L. plantarum* is effective for alleviating atopic dermatitis symptoms in adults owing to its immunomodulatory effects [9]. *L.*

plantarum against UVB-induced photoaging in human dermal fibroblasts and hairless mice [10]. Meanwhile, *L. plantarum* possesses various pharmacological properties, such as anti-cancer and anti-colitis [11].

We focused on the screening of skin care function from various probiotics for enhancing collagen synthesis and the gene expression of serine palmitoyltransferase small subunit A (SPTSSA). In this study, we sought to investigate that *L. plantarum*-GMNL6 play a role in maintenance skin and wanted to develop *L. plantarum*-GMNL6 as skin care products for the management of skin conditions.

Materials and Methods

Cells, bacteria, and reagents

Human skin fibroblast HS68 cells and mouse melanoma B16F10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50µg/ml gentamicin, and 10% fetal bovine serum, at 37°C in 5% CO₂. *Staphylococcus aureus*, *Propionibacterium acnes*, *Enterococcus faecium*, *L. plantarum*-2, *L. plantarum*-GMNL6, *L. rhamnosus*, *L. paracasei*-2, *L. paracasei*-1, *L. fermentum*-3, *L. fermentum*-2, *L. fermentum*-1, and *L. acidophilus* are available from GenMont Biotech Inc. (Tainan, Taiwan).

Collagen synthesis assay

HS68 cells were seeded to 6 well plate (2×10⁵ cells/well), washed twice with phosphate buffered saline (PBS), and replaced with serum free medium, and treated with various heat-killing bacteria (5×10⁸ cells/ml) after 24 h. The supernatant was collected and then analyzed using a Procollagen type I C-peptide EIA kit (TAKARA Bio, Inc, #MK101, Kusatsu, Japan).

Detection of neural amine synthase gene

HS68 cells were seeded to 6 well plate (2×10⁵ cells/well), and treated with various heat-killing bacteria (5×10⁸ cells/ml) after 24 h. The cell extracts were collected, RNA was extracted and converted into cDNA, and the serine palmitoyltransferase small subunit A (SPTSSA) and the β-actin were quantified by real-time-PCR analysis.

Purification of Lipoteichoic acid (LTA) and Peptidoglycan (PGN)

L. plantarum-GMNL6 was incubated overnight at 37°C. The cells were disrupted using an ultrasonic disruptor (ultrasonic instrument model W-220F) and then centrifuged. After centrifugation, the bacteria were reconstituted with a mixture of chloroform-methanol-water (1:1:0.9), and the aqueous phase containing LTA was collected. The solution was

then injected onto Octyl-Sepharose CL-4B column (Sigma) and Q-Sepharose column. The LTA was purified, and stored at -20°C.

The lactic acid bacteria were collected by centrifugation. After washing three times with PBS, 50 g of lactic acid bacteria (wet weight) was broken by an ultrasonic breaker to prepare a crude extract. After centrifugation at 8,000 rpm for 30 minutes, the supernatant was collected and then washed three times with PBS, and centrifuged (8,000 rpm, 30 minutes, 4 °C), and this procedure was repeated three times. Finally, the precipitates were reacted with 100 mg / ml RNase and 50 mg/ml DNase for 18 h, and then 200 mg/ml trypsin was added, and then reacted at 37°C for 18 h. The precipitates were treated with 5% trichloroacetic acid (TCA). The TCA extract was dried to prepare a peptidoglycan powder.

Determination of melanin content

The B16F10 cells were harvested and washed twice with PBS. The pelleted cells were lysed in cold lysis buffer (20 mM sodium phosphate pH 6.8, 1% Triton X-100, 1 mM PMSF, and 1 mM EDTA). After centrifugation at 15 000 g for 15 min, the melanin pellets were dissolved in Soluene-350 (Perkin-Elmer, Waltham, MA, USA) for 15 min at 100 °C. The absorbance at 400 nm was measured [12]. The protein content in each sample was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA).

The biofilm formation assay

S. aureus was incubated overnight at 37°C. The different concentrations of LTA were treated in to 96 well that had been incubated *S. aureus* (2 ×10⁷ cfu/well) for 24 hours. The supernatant was removed and washed twice with PBS. The biofilm was fixed with ethanol for 15 min and stained with 0.1% crystal violet. Then, crystal violets were dissolved in acetic acid. The absorbance at 590 nm was measured.

Study design

A 2-month study was performed. The study was approved by the Ethics Committee of Pingtung Antai Hospital and performed in accordance with the relevant guidelines and regulations. The trial was registered at Clinical Trial.gov with approval NO. NCT03350893. A total of 15 females (Age from 25-50 years old) were recruited. At first visit, participants underwent a baseline examination, including face quality diagnosis, moisturizing test and skin color test. The left face of the participants used base cream and the right face of the participants used base cream including heat-killed *L. plantarum*-GMNL6 (1×10⁹ cells/g cream). The test was used once in the morning

and evening for 2 months and every month the face quality (Visia Complexion Analysis, Canfield, USA), moisturizing test and skin color test was measured. Corneometer® CM 825 (Courage+Khazaka electronic GmbH, Köln, Germany) and Skicon 200 EX® (IBS Co., Hamamatsu, Japan) were used to determine the hydration level of the skin surface. Derma-Spectrometer (Cortex Technology, Hadsund, Denmark) was used to determine the E index (erythema) and M index (melanin) of the skin surface [13]. DNA was extracted from the heads of cotton swabs using Quick-DNA Fungal/Bacterial Kit (Zymo research, D6005, CA, USA). Full length 16S rDNA were amplified using forward primers 27F containing the sequence “AGRGTTYGATYMTGGCTCAG” and reverse primers 1492R containing the sequence “RGYTACCTTGTTACGACTT”. At the same time, indexed adapters were added to the ends of the 16S rDNA amplicons using SMRT cell Template Prep Kit (Pacific Biosciences of California, Inc., CA, USA) to generate indexed libraries ready for downstream sequencing on Sequel SMRT cell. DNA libraries were validated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit 2.0 Fluorometer. DNA libraries were multiplexed and loaded on PacBio Sequel instrument according to manufacturer’s instructions (Pacific Biosciences of California, Inc., CA, USA). PacBio 16S reads were processed using SMRT Analysis.

Statistical analysis

All data were expressed as mean \pm standard deviation (SD). The unpaired, two-tailed Student’s t test was used to determine differences between groups. Any P value less than 0.05 is regarded statistically significant.

Results

L. plantarum-GMNL6 displayed beneficial effects on skin care

In order to find out the optimal probiotics, the various potentially probiotics were treated with HS68 cells by using collagen synthesis and the gene expression of serine palmitoyltransferase small subunit A (SPTSSA) assays. As shown in Figure 1A, the collagen synthesis was significantly increased after *E. faecium* and *L. plantarum*-GMNL6 treatment. The collagen and ceramide are involved in skin rejuvenation. Ceramide is a lipid that exists in the intercellular space of skin cells. The sphingosine and fatty acid constitute ceramide which is the most important and highest proportion of lipids of the skin, with a total content of about 40-50% of the intercellular lipids. Ceramide distributed in the

intercellular space and closely maintains the lipid integrity to form a natural waterproof membrane. Ceramide prevents the evaporation of water, allows the keratinocytes to lock in moisture. Serine palmitoyltransferase is the rate-limiting step in ceramide biosynthesis. *L. plantarum*-GMNL6 had the best ability to stimulate the gene expression of SPTSSA compared with other probiotics (Fig. 1B). Dependent on the two assays, *L. plantarum*-GMNL6 was selected in the subsequent analyses.

The potential components of *L. plantarum*-GMNL6 involved the beneficial effects on skin care

Because we used the death bacteria to treat the cells, we proposed that the cell wall components played an important role in collagen synthesis and SPTSSA gene expression. The lipoteichoic acid (LTA) and peptidoglycan (PGN) are the major cell wall components of *Lactobacillus* that had been demonstrated the pivotal components for beneficial effects on host [14]. First, the collagen synthesis and SPTSSA gene expression were used to evaluate the function of cell wall components (LTA and PGN). Although PGN slightly increased collagen synthesis and SPTSSA gene expression in HS68 cells, the LTA of *L. plantarum*-GMNL6 had significant effects on collagen synthesis and SPTSSA gene expression (Fig 2 A and B). Herein, the results suggest that LTA of *L. plantarum*-GMNL6 involved in skin health. Meanwhile, mouse B16F10 melanoma cells were used to investigate the anti-melanogenic activity of the LTA of *L. plantarum*-GMNL6. The melanin content after B16F10 cells treated with various concentrations LTA are shown in Fig 2 C. *Staphylococcus aureus* is commonly found on the surface of skin. The LTA of *L. plantarum*-GMNL6 significantly inhibited the biofilm formation of *S. aureus* in a dose-dependent manner (Fig. 2D).

A heat-killed *L. plantarum*-GMNL6 in clinical trial

The study design is shown in Fig. 3. A total of 15 females (Age from 25-50 years old) were recruited. At first visit, participants underwent a baseline examination, including face quality diagnosis, moisturizing test and skin color test. The left face of the participants used base cream and the right face of the participants used base cream including heat-killed *L. plantarum*-GMNL6 (1×10^9 cells/g cream). We hypothesized that heat-killed *L. plantarum*-GMNL6 could interact with skin microbiota and acquire different microbial communities that might finally result in successful skin health. To examine the relationship between the skin microbiota and

heat-killed *L. plantarum*-GMNL6 treatment, we performed 16S rDNA-based sequencing to analyze the bacterial abundance and composition in the human skin. *Propionibacterium* was significantly lowered in the *L. plantarum*-GMNL6 treatment. *Streptococcus* and *Staphylococcus* also showed higher abundance compared with no treatment. Furthermore, the test was used once in the morning and evening for 2 months and every month the face quality, moisturizing test and skin color test was measured. Corneometer® CM 825 and Skicon 200 EX® were used to determine the hydration level of the skin surface. Derma-Spectrometer was used to determine the E index (erythema) and M index (melanin) of the skin surface. As shown in Table 1, the

hydration levels of the heat-killed *L. plantarum*-GMNL6 treated skin surface were significantly higher than those of the control group. The hydration levels of the skin surface were similar by using different instruments. Meanwhile, the heat-killed *L. plantarum*-GMNL6 had the ability to reduce the erythema and melanin (Table 1). Furthermore, by using Visia to detect the face quality, the wrinkles, texture, and UV spots had been significantly improved after heat-killed *L. plantarum*-GMNL6 treatment (Table 2). The porphyrins of the treated face were reduced compared with the non-treated face. Taken together, the cream that added heat-killed *L. plantarum*-GMNL6 can improve the face quality.

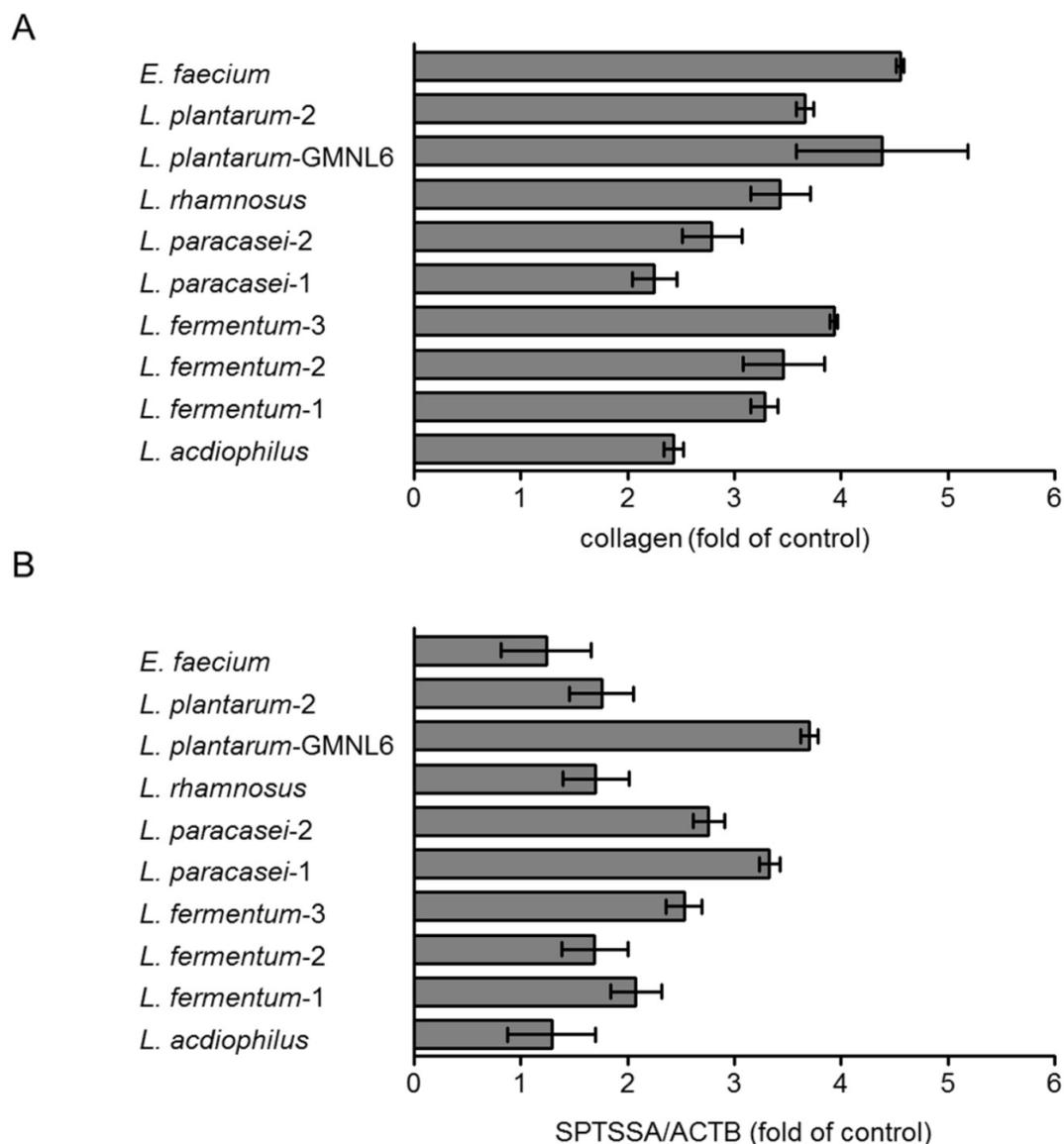


Figure 1. Effects of various probiotic on skin health. HS68 cells were treated with various probiotics (5×10^8 cells/ml) after 24 h. (A) The supernatant was collected and then analyzed collagens. (B) The cell extracts were collected, RNA was extracted and converted into cDNA, and the serine palmitoyltransferase small subunit A (SPTSSA) and the β -actin were quantified by real-time-PCR analysis.

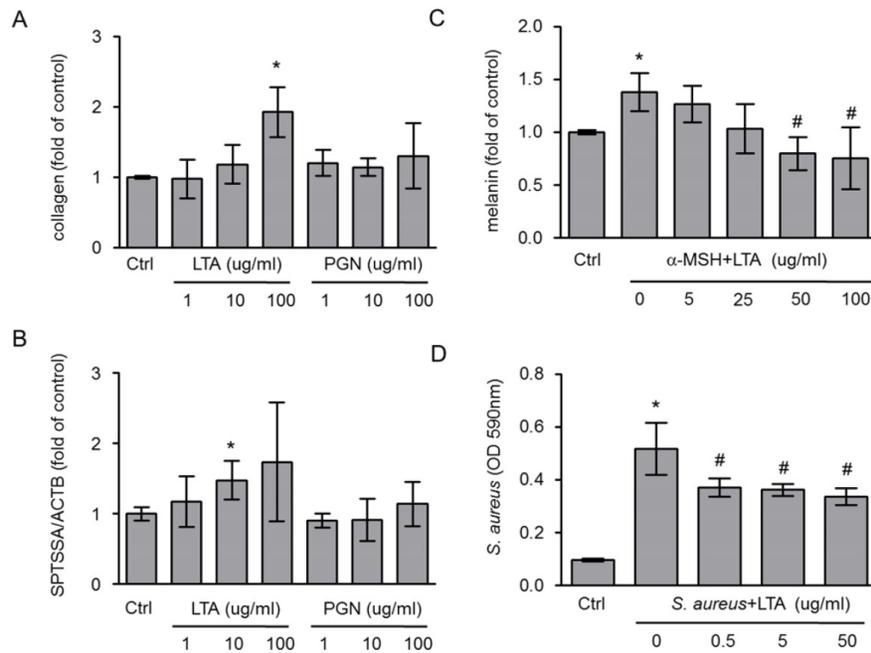


Figure 2. The collagen synthesis, the gene expression of SPTSSA, melanogenesis, and biofilm formation were measured after lipoteichoic acid (LTA) and peptidoglycan (PGN) of *L. plantarum*-GMNL6 treatment. Effect of LTA and PGN on (A) cellular collagen synthesis and (B) the gene expression of SPTSSA. (C) Effect of lipoteichoic acid (LTA) and peptidoglycan (PGN) on cellular melanin content. (D) The inhibition of biofilm formation of *S. aureus* was measured after LTA and PGN treatment. (n = 6, data are mean ± SD. * P < 0.05, # P < 0.01).

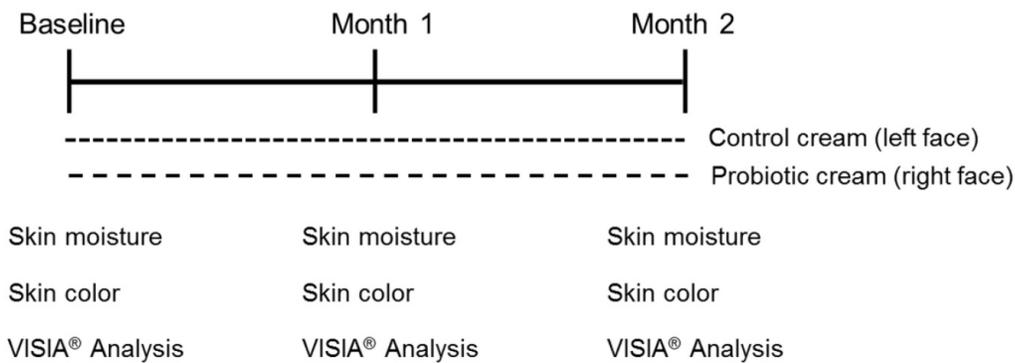


Figure 3. Recruitment of participants and the study design. The cream containing probiotics or not was performed a total of 2 months. All participants were requested to collect data by using Corneometer® CM 825, Skicon 200 EX® Derma-Spectrometer, and VISIA®.

Table 1. The effects of *Lactobacillus plantarum*-GMNL6 on human skin health.

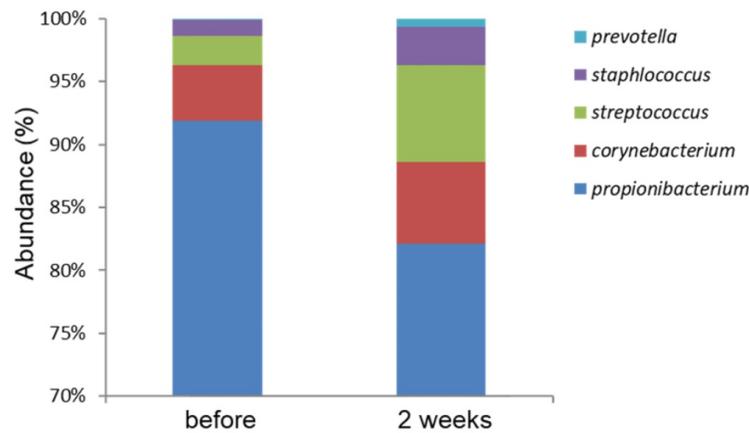
	left face (control)			right face (GMNL-6)		
	baseline	1M	2M	baseline	1M	2M
	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE
Skin moisture						
Skicon	209.7 ± 27.4	191.4 ± 17.5	215.9 ± 33.3	179.6 ± 18.8	215.4 ± 19.9 ^c	224.1 ± 30.5
Corneometer-CM825	64.3 ± 3.9	62 ± 3.8	57.5 ± 4.8	61.5 ± 3.7	67.5 ± 3.6 ^c	64.1 ± 3.7 ^c
Skin color						
E index (erythema)	11.5 ± 0.9	11.5 ± 1	10.4 ± 0.6	11.3 ± 1.1	8.7 ± 0.7 ^c	8.6 ± 0.8 ^{b, c}
M index (melanin)	31.9 ± 0.7	31 ± 0.6	29.6 ± 0.5 ^a	32.5 ± 0.8	31.5 ± 0.6	30.1 ± 0.4 ^b

a: comparing with baseline in left face; P < 0.05 was considered statistically significant.
 b: comparing with baseline in right face; P < 0.05 was considered statistically significant.
 c: comparing with left face at the same time period; P < 0.05 was considered statistically significant.

Table 2. The effects of *Lactobacillus plantarum*-GMNL6 on human skin health.

	left face (control)			right face (GMNL-6)		
	baseline	1M	2M	baseline	1M	2M
	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE
spots	102.2 ± 10.0	104.3 ± 9.8	102.8 ± 9.4	107.8 ± 10.0	104.1 ± 8.7	106.1 ± 9.9 ^a
wrinkles	14.1 ± 1.3	15.5 ± 1.7	19.3 ± 1.9	17.8 ± 2.7	12.4 ± 1.7	15.3 ± 1.7
texture	571.8 ± 103.5	554.2 ± 99.1	674.8 ± 132.9	512.3 ± 105.3	494.6 ± 89.2 ^b	582.9 ± 119.0 ^b
pores	808.2 ± 81.9	845.2 ± 86.1	860.7 ± 88.7	830.7 ± 87.7	840.5 ± 85.0	876.3 ± 91.5
UV spots	245.3 ± 16.9	252.9 ± 17.1	240.4 ± 12.6	243.3 ± 16.5	226.0 ± 16.5 ^{a, b}	225.5 ± 16.8
brown spots	271.2 ± 16.7	276.4 ± 17.7	270.2 ± 12.7	278.9 ± 18.7	280.1 ± 19.1	268.1 ± 15.3
red areas	134.2 ± 17.1	124.3 ± 13.9	126.7 ± 18.7	128.1 ± 18.9	121.8 ± 14.5	121.5 ± 20.1
porphyrins	981.4 ± 212.1	995.0 ± 222.3	1089.8 ± 242.3	1057.6 ± 240.1	955.7 ± 209.4	1009.7 ± 230.2

a: comparing with baseline in right face; P < 0.05 was considered statistically significant.



Abundance (%)	before	2 weeks	P value
<i>Propionibacterium</i>	91.9	82.1	0.036
<i>Corynebacterium</i>	4.4	6.5	0.224
<i>Streptococcus</i>	2.3	7.7	0.081
<i>Staphylococcus</i>	1.3	3.1	0.062
<i>Prevotella</i>	0.1	0.6	0.185

Figure 4. Comparison with the skin microbiomes before and after using *L. plantarum*-GMNL6 treatment. The relative abundance of five major genera in skin microbiomes is compared before and after using *L. plantarum*-GMNL6 treatment. Statistically significant P-values are shown in red.

Discussion

Probiotics are considered important roles in skin health, wellbeing, and microbiome-associated skin disease. Therefore, we explored the possibility that probiotics associated with skin health. Supplementation of the *L. plantarum*-GMNL6 to cell improved the collagen synthesis and moisturizing. Although probiotic cell wall components have been demonstrated to be related to probiotic function [15], very few evidences proposes that they contribute for

skin care. In the present study, the LTA of *L. plantarum*-GMNL6 was involved in improvement of skin condition, including collagen synthesis, moisturizing, melanogenesis, and anti-biofilm formation. In human studies, some people have been facing a lot of ordeals at skin condition. After two months *L. plantarum*-GMNL6 treatment, we observed that some skin conditions had been improved in human.

Unlike LTA from other Gram-positive bacteria, LTA from *L. plantarum*-GMNL6 acts as cosmetic

regulator substance. In accordance with previous observations, the LTA of *Lactobacillus* has multi-functions, including anti-inflammatory, improvement of diarrhea, and blood glucose control [7]. Previously, we found that an extract of *R. sphaeroides* inhibited proinflammatory cytokines and enhanced mucosal immune balance in a dextran sodium sulfate (DSS)-induced colitis model [3, 16]. We also demonstrated the extract of *R. sphaeroides* as an anti-melanogenic agent with the capacity to ameliorate α -MSH induced-hyperpigmentation [17].

The bacteria produce compounds that are widely used in cosmetic and pharmaceutical applications. Microbe-based therapies cosmetic has been noticed. The mitogen-activated protein kinase (MAPKs) signaling pathway involved in the melanogenesis. The activation of protein kinase B (AKT) is responsible for suppression for melanin synthesis [18]. The LTA of *L. plantarum*-GMNL6 may affect the signaling to reduce the melanogenesis. The people *L. plantarum*-GMNL6 had better skin conditions after 1 month *L. plantarum*-GMNL6 treatment (Fig. S1). Further work is warranted to elucidate the molecular mechanism of *L. plantarum*-GMNL6 for hypopigmentation. These findings point out that LTA of *L. plantarum*-GMNL6 might contribute to its therapeutic effect on anti-melanogenesis.

Supplementary Material

Supplementary figure.

<http://www.medsci.org/v18p1114s1.pdf>

Acknowledgements

This work was supported by Higher Education Sprout Project, Taiwan.

Author Contributions

W.H.T., C.H.C. and Y.J.C conceived, designed and revised the experiments; W.H.T, C.H.C, Y.J.C, and C.G.L. performed the experiments; W.H.T. and C.H.L analyzed the data; W. H.T. and C.H.L contributed reagents/materials/analysis tools; C.H.L wrote the paper.

Competing Interests

The authors have declared that no competing interest exists.

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