International Journal of Clinical and Diagnostic Research, 2021, Vol. 9, No. 1, 18-28
Available online at http://pubs.sciepub.com/ijcdr/9/1/3
Published by Science and Education Publishing
DOI:10.12691/ijcdr-9-1-3

The Novel Use of Postbiotics of TYCA06/AP-32/CP-9 in the Amelioration of Atopic Dermatitis and Improvement of Skin Care - A Clinical Study

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Received February 12, 2021; Revised March 18, 2021; Accepted March 26, 2021

Abstract The development of human society has created many synthetic materials for better convenience; however, these innovated products usually produce unnatural side chemicals in the environment. The skin is the first barrier of defense against pathogens and toxicants, so the health of skin is important not only on facial appearance but also on physical health. Many people live a busy life and the habit of refined diet and over-cleaning does not improve the quality of life. In contrary, the overuse of detergents and antibiotics may cause skin dysbiosis, and result in a sensitive skin, aging skin, or, moreover, atopic dermatitis. In this study, the postbiotic blend of three probiotic strains, TYCA06/AP-32/CP-9 (TAC), displayed the growth inhibition against *Staphylococcus aureus*, and the stimulation of the anti-inflammatory cytokine *in vitro*, such as transforming growth factor (TGF)-β and interleukin (IL)-10. This TAC formula enhanced the wound healing in keratinocytes HaCaT cell culture and increased moisture content on skin *in vivo*. The TAC formula was further incorporated into a cosmetic lotion, and the human clinical trial was carried out by applying this lotion on volunteers' face or hand with atopic dermatitis (AD). The result showed the TAC lotion improved the moisture score, inflammation index, and brightening score of the skin. In addition, this TAC lotion also improved the wound repair of cracked skin in volunteers with AD. Moreover, this TAC lotion reduced the number of the speckle and wrinkles on facial skin. Taken together, this postbiotic blend of TAC has beneficial effects on skin health, and is able to ameliorate the red and itchy skin in AD patients.

Keywords: *postbiotics, skin, atopic dermatitis*

Cite This Article: Hsieh-Hsun Ho, Ching-Wei Chen, Yu-Fen Huang, Yi-Wei Kuo, Jia-Hung Lin, Jui-Fen Chen, Chen-Hung Hsu, Tsai-Hsuan Yi, Leong-Perng Chan, and Chia-Hua Liang, "The Novel Use of Postbiotics of TYCA06/AP-32/CP-9 in the Amelioration of Atopic Dermatitis and Improvement of Skin Care - A Clinical Study." *International Journal of Clinical and Diagnostic Research*, vol. 9, no. 1 (2021): 18-28. doi: 10.12691/ijcdr-9-1-3.

1. Introduction

Environmental stimuli derived from the microbiome and a material is referred as allergens in general, and provokes inflammation responses when it comes in contact with the skin [1]. Atopic dermatitis (AD) is a long-term type of inflammation of the skin, and affects about 20% of people at some point in their lives [2]. The cause of AD remains unclear and may be a combination of different factors, e.g. genetics, hygiene, allergens, pathogens, and even hard water [3]. Although treatments can ameliorate the severity and frequency of symptoms, e.g. itchy, red, swollen, and cracked skin, there is no known cure for AD. The growth of *Staphylococcus aureus* on skin shows an extreme prevalence in those with atopic dermatitis [4], and abnormalities of the epidermal barrier in AD decline the protection against *S. aureus* entry into the dermis. The colonization of *S. aureus* can trigger the expression of cytokine, and result in increased inflammatory and itchiness [5]. Scratching the affected areas increases the risk of skin infections, and antibiotics may be needed if a bacterial infection develops.

The medical use of probiotics and prebiotics appear to be effective in reducing the incidence of skin problems. The role of probiotic product in acne, wound healing, and photoprotection is promising; however, their role in atopic dermatitis treatment is controversial [6]. At the beginning, the approach focused on the balance of the microbiota in intestine, and the immunomodulatory effects which suppress the Th2 response and stimulate Th1 cytokines.

The oral supplementary of probiotics is found to prevent or treat allergic diseases, including atopic dermatitis, by modulation of the intestinal microbiota [7]. Recently, more and more studies have focused on the balance of the microbiota on skin, and the support of the skin's immune system which is affected by age, climate, hygiene or antibiotic consumption [8]. Skin microbiota compositions in AD showed distinct differences compared with healthy skin, and the restoration of host-microbiota homeostasis could be future strategies [9].

Although only few clinical trials were done for the therapeutic effect of probiotics on AD in human [10], the concept of commensal homeostasis promotes the topical use of probiotics currently. Skin care is delicate and necessary in AD patients, so the market of AD cosmetics or products for sensitive skin is growing rapidly. In order to apply the beneficial effects of probiotics on skin into daily life, many cosmetic products have tried to incorporate live probiotic bacteria in their ingredients. However, it brings up the doubt that if probiotics are still alive in the cosmetics that have preservatives. In fact, 90 percent of cosmetic products that carry “probiotic labels” actually have “postbiotics”, which consists of metabolic byproducts from probiotics [11]. In other words, the efficacy of these cosmetics, if any, actually comes from the postbiotics, rather than probiotics itself.

This is a novel study focused solely on the effect of postbiotics on skin health. Based on the result of growth inhibitions against *S. aureus* and inductions of TGF-β/IL-10 *in vitro*, the postbiotic blend of three probiotic strains, TYCA06/AP-32/CP-9, was created as the TAC formula. The effect of TAC on wound healing was performed in HaCaT cells, and two concentrations of this TAC formula were tested for the effect of moisturizing on skin *in vivo*. This TAC formula was further incorporated into a cosmetic lotion, and values of moisture score, sebum content, inflammation index, and brightening score were compared with the control lotion without TAC. The amelioration of atopic dermatitis was evaluated in AD patients, and the improvement of skin speckle and wrinkle was investigated in volunteers.

2. Materials and Methods

2.1. Isolation, Identification and Culture of Bacterial Strains

Eight lactic acid bacteria (LAB) strains were isolated at Glac Biotech Co., Ltd., including *Lactobacillus salivarius* AP-32, *L. acidophilus* TYCA06 and *L. reuteri* GL-104 were isolated from the intestines of healthy individuals. *Bifidobacterium animalis* subsp. *lactis* CP-9, *B. longum* subsp. *infantis* BLI-02, *B. breve* Bv-889, *L. gasseri* AI-88 and *B. bifidum* Bf-688 were isolated from breast milk. All strains were identified using their entire 16S rDNA sequences as described previously [12-14] and using the National Center for Biotechnology Information nucleotide Basic Local Alignment Search Tool (BLAST). All strains were each successfully activated three times by incubation in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Detroit, USA) at 37°C for 24 h and then centrifuged at 10,000×*g* at 4°C for 10 min. The supernatant will be collected separately and named lactobacillus postbiotics. TAC postbiotics were 1:1:1 mixed with TYCA06, AP-32 and CP-9.

2.2. Antimicrobial Activity Assay for Live LAB

The modified method of overlay agar [15-16] was used to test the antimicrobial activities of the LAB. The LAB was cultured on the basal layer of Lactobacilli MRS agar (Creative, Taiwan) in the form of a 2-cm-wide stripe across the plate. The plate was incubated in either aerobic, facultative anaerobic, or anaerobic conditions at 37°C for 24 h. The LAB/MRS plate was then overlaid with the top layer of melted NA agar (Merck, Louis, USA), then applied the *S. aureus* (BCRC 12154 from Bioresource Collection and Research Center, Hsinchu, Taiwan) by sterile swab after NA solidification. The overlay plate was incubated under aerobic conditions at 37°C for 20 h. After incubation, the inhibition zone against the pathogen was measured and quantified using a semi-quantitative scale as following: (+/-), < 2 cm growth inhibition; (++), < 3 cm growth inhibition; (+++), < 4 cm growth inhibition; (++++), < 5 cm growth inhibition; (+++++), < 8.5 cm growth inhibition; and (++++++), ≥8.5 cm growth inhibition.

2.3. Antimicrobial Activity Assay for LAB Postbiotics

S. aureus was cultured in a Nutrient broth or agar (NB or NA) (Merck, Louis, USA) under the 37°C for 20 h, and adjusted to the concentration of 1×10^9 CFU ml^{-1}. 100 µl of pathogenic culture was mixed with 4.9 ml LAB postbiotics (4%), and incubated for 6 h. The culture was three serial diluted after the master dilution of 1:10,000, and plated on the appropriate agar for 24 h. The inhibition rate was calculated as {inhibition rate (%) = [1- (colony count of the test group/colony count of the control group)] * 100}.

2.4. Assay for Cytokines and Proliferation of PBMC

Human denfritic cells (DCs) were generated from peripheral blood mononuclear cells (PBMCs). PBMCs were obtained from blood samples of two subjects with atopic dermatitis by centrifugation through a Ficoll-Hypaque gradient (Pharmacia, Sweden). The clinical research was approved by the ethics committee of the Antai Medical care corporation Anti Tian-Sheng Memorial Hospital (IRB No. 20-027-A). Anti-inflammatory cytokines, i.e. IL-10, and TGF-β, were assayed in culture supernatants from co-cultures of DCs and *Lactobacillus* sp. postbiotics (4%) were mixed at a ratio of 1:10 and incubated at 37°C for 2 days. As a positive control, cells were treated with phytohemagglutinin (PHA, 1 µg/ml, Sigma-Aldrich) to induce cytokines, while untreated cells and medium only were treated as the control. Cytokines were assayed with an enzyme-linked immunosorbent assay (ELISA) kit by following the manufacturer’s instructions (R&D Systems Minneapolis, MN, U.S.A.; eBioscience, San Diego, CA, U.S.A.).

2.5. Cell Culture

HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Gibco, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (EMD millipore, U.S.A) and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, U.S.A.). Cells were incubated at 37°C in 5% CO_2.

2.6. Assay of Cell Viability

HaCaT cells (2×10^4 cell/ml) were seeded in 96-well plates. In the following day, cells were treated with DMSM and TAC postbiotics (0-1%). After incubation for 24 or 48 h with cells, 10 µl of MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] solution (10 mg/ml) was added to each well, and cells were incubated for an additional 4 h at 37°C. The medium was removed and replaced with 150 µl DMSO, and the plate was incubated for 30 min at room temperature with shaking. Absorbance was measured at 570 nm using a spectrophotometer

2.7. Assay of Scratch Wound Healing

HaCaT cells were seeded in 48-well plates at a density of 1×10^6 cells/well for 24 h. A scratch was made on the cell monolayer by drawing a pipette tip across the well. The culture medium was supplemented with medium DMEM or with TAC postbiotics dose-dependently. At time 0 and 24 h post-treatment, wound closure was captured using an Olympus IX70 microscope (Tokyo, Japan) equipped with a digital camera at 40× magnification. The distance migrated was measured using the Image J software (Lviv, Ukraine), and calculated by comparing between the initial and final width of the scratch.

2.8. Quantification of Free Amino Acids

To evaluate the variation in the amino acid concentration, samples were analyzed by HPLC (A-10 Solvent & Sample module, A-10 PDA detector and A-10 RI detector Altus, PerkinElmer, U.S.A), after a derivatization using an AccQ-Tag chemistry package kit (Waters Corp., Milford, MA, U.S.A.) according to the method described by Montanari et al [17]. The separation of amino acids was performed using an AccQ-TagTM column (3.9 × 150 mm; Waters Corp.) at 30°C using mobile phase A (ddH_2O), mobile phase B (100 mL of AccQ-Tag Eluent (Waters Corp., Milford, MA, U.S.A.), diluted 1:10 with H_2O for chromatography (Sigma-Aldrich, St. Louis, MO, USA) and mobile phase C (100% acetonitrile for chromatography (Sigma-Aldrich, St. Louis, MO, U.S.A.) at a flow rate of 1 mL/min. Amino acid standard H (Waters Corp., Milford, MA, U.S.A.) was used as the standard. The fluorescent detector was set at an excitation wavelength of 247 nm. Under the adopted conditions, good separation of the amino acids was obtained with the exception of the couples of histidine + glutamine and serine + asparagine, which were co-eluted in single peaks. Tryptophan was not detectable with this protocol.

2.9. Study Design-double-blind, Placebo-control Trail Study

The clinical research was approved by the ethics committee of the Antai Medical care corporation Antai Tian-Sheng Memorial Hospital (IRB No.20-027-A). All subjects recruited in this trail returned the written consent forms before the study.

2.9.1. Human Skin Hydration Detection in a Short Time

Ten subjects were recruited and asked to apply 0%, 0.2% or 0.4%, of placebo/TAC lotion (formula showed as Table S1) on different visits. The skin hydration was detected at two time points, right after the application at 0 h and 3h after by intelligent skin analysis system (ES3100, Yi Li Mei technology, China). Every visit was at least one-week apart, and subjects were only allowed to wait in certain indoor spaces during the 3 h waiting period. Note that participants and the experimental operators, who conducted the measurement, were not aware of the content of the lotion.

2.9.2. Human Skin Efficacy Assessment in a Long Time

Each subject was asked to apply TAC lotion (named A lotion) on their right face and placebo lotion (named B lotion) on their left face (formula showed as Table S1). Twenty subjects were recruited and were informed to apply twice per day (3-5 mg/cm^2 once) for 4 weeks. Ordinary cosmetics usage was permitted but the change of usage was not allowed during the intervention period other than the provided product/placebo. Outdoor activities (sun exposure) and the usage of sunscreen lotion were not limited in this study. Each subject was required to undergo skin condition inspection on week 0, 1, 2 and 4. Every subject should wash face with water and wait for 30 min at room temperature environment (25°C, humility 55 ± 5%) before instrument detection.

Skin hydration (Corneometer CM825, Courage + Khazaka Electroni, Germany), sebum (Callegari 1930, Italy), inflammation detection and brightness/color (Chroma Meter MM-500, Minolta, Japan) of each subject's upper cheek were measured. The degree of improvement in inflammation detection or skin brightness are positively correlated with the decrease of a^* value or increase of L^*. VISIA® Complexion Analysis (VISIA® Complexion Analysis, U.S.A.) was also employed to measure the skin inflammation, pores and wrinkles of full face. The results were presented as the mean value and the relative percentage (%) to the baseline.

2.10. Effect of TAC Lotion on Skin Wound

The skin of upper arm is the sensitive to stimulus, and the area was selected to test for the skin irritation caused by TAC lotion. About 20 µl of TAC lotion/ddH_2O was prepared on the medical film (1.0 x 1.0 cm) (3M™ Tegaderm™, Minnesota, U.S.A.) and attached to the subjects` upper arm skin with waterproof bandage (4.4 x 4.4 cm) (3M™ Tegaderm™, Minnesota, U.S.A.) for 24 h. Skin irritation such as erythema, papules, blisters and

other irritation reactions was observed and evaluated for 48 h after removing the taps. Once the irritation occurred at any time during the period, the test would be halted and the film would be removed immediately.

2.11. Statistical Analysis

Data are presented as the mean ± standard error of mean (SEM) obtained from three repeats of per sample. All values from different treatment groups were compared using Tukey's post hoc test through GraphPad Prism, as $p < 0.05$ was considered statistical significance. The values of the measurement results for skin parameter were transfer to standard deviation (%) and set 100% as the initial value. The comparison of measurement results for skin parameters among groups and between groups was analyzed by one-way repeated measurement ANOVA and one-way ANOVA, respectively, followed by Tukey's post hoc test through GraphPad Prism, as $p < 0.05$ was considered statistical significance.

3. Results

3.1. The Determination of LAB Postbiotics by the Antibacterial Activity against *S. aureus* and Stimulation of Cytokine Level *in vitro*

S. aureus is frequently isolated from the skin of AD patients [18]. The resistance of LAB strains against *S. aureus* was the first priority of the screening. In the antibacterial assay (Figure 1A), Bf-688 performed the best anti-pathogenic ability (≥ 8.5 cm growth inhibition), followed by TYCA06 and Bv-889 (5~8.5 cm growth inhibition), and GL-104 showed the lowest value (< 2 cm growth inhibition). There are many mechanisms for probiotics to resist pathogen and their postbiotics are one of the strategies [19]. To clarify whether their postbiotics can inhibit *S. aureus*, sterile postbiotics of these trains were collected and co-cultured with *S. aureus* for 6 h. The postbiotics of three strains, CP-9, AP-32, and TYCA06, showed the best ability in the resistance of *S. aureus*, which displayed inhibition rates of 30.37 ± 14.1%, 30.19 ± 19.1% and 30.19 ± 19.1%, respectively (Fig. 1B), as compared with that of control (0.0%, $p < 0.05$, $p < 0.05$ and $p < 0.05$, respectively). The *S. aureus* inhibition rate in postbiotics was different from the result in overlay agar (Figure 1A and Figure 1B), which indicated that the antibacterial substances metabolized by each strain were not the same. Furthermore, the TGF-β and IL-10 levels in PBMC of atopic dermatitis patients were used to evaluate the anti-inflammatory cytokines expression. The postbiotics of all LAB strains showed significantly higher TGF-β and IL-10 expression than control ($p < 0.001$, respectively), except for Bv-889 and Bf-688 (Figure 1C and Figure1D). The result revealed that anti-inflammatory cytokines of LAB postbiotics was not exactly the same as the antibacterial effect against *S. aureus*. Based on those results (Figure 1), the postbiotics of these three strains (TYCA06, AP-32 and CP-9), which showed the highest *S. aureus* inhibition rate and good anti-inflammatory level, and named TAC for the further study.

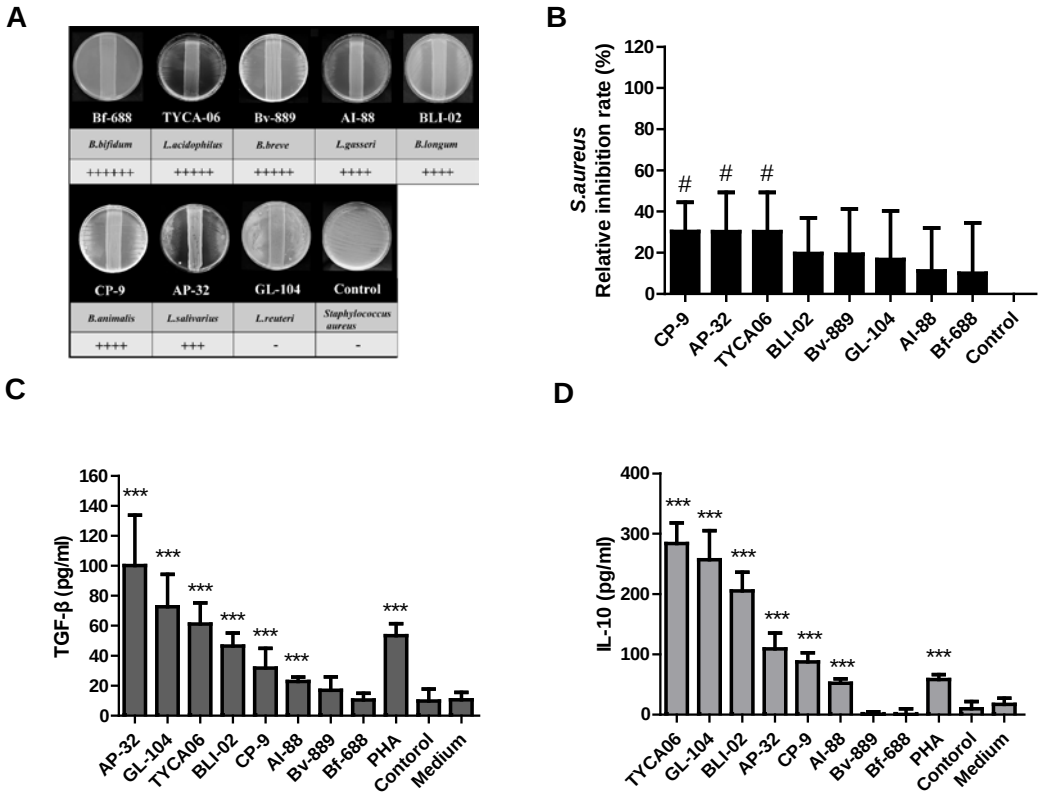

Figure 1. Eight LAB strains and their postbiotics screening on anti-*S.aureus* and anti-inflammatory cytokine repression. (A) Bacteriostatic activity of different viable LAB strains against *S. aureus* determined by measuring the inhibition area. Scale: (-), 0 cm growth inhibition; (+++) < 4 cm growth inhibition; (++++) < 5 cm growth inhibition; (+++++) < 8.5 cm growth inhibition; (++++++) > 8.5 cm growth inhibition. (B) *S. aureus* relative inhibition rate in eight 4% LAB postbiotics for 20 min. (C) TGF-β and (D) IL-10 inflammatory cytokine expression. PHA: phytohemagglutinin as the positive control; untreated cells and medium: MRS broth only as the control; $^{\#}p < 0.05$ vs. control; *** $p < 0.001$ vs. control

3.2. TAC Postbiotic Amino Acid Composition and Its Effects on Basal Layer of Human Keratinocytes

LAB postbiotics gained special importance in the treatment of the inflammatory response [20]. To further examine whether the TAC postbiotics could affect the re-epithelization, the wound healing assay was applied on human keratinocytes HaCaT cells and compared the migration after 24 h with/without 0.3125% TAC treatment (Figure 2). A better healing ability was displayed in the medium with TAC treatment (Figure 2D) and showed a migration of 62.8 ± 7.7% (Figure 2E), which was significantly higher than in control treatment (52.2 ± 1.8%, $p < 0.05$) (Figure 2B and Figure 2E). Furthermore, amino acid compositions of TAC postbiotics were analyzed by HPLC (Table S2), and the result showed the contents of alanine, glutamate and leucine were the most abundant, which presented 18.37%, 12.61% and 12.59%, respectively.

3.3. Effects of the Different Concentrations of TAC Postbiotics Lotion on Human Skin Hydration in a Short-term Test

Skin hydration and moisturizing play a key role for AD patients [21]. In this study, three concentrations, 0%, 0.2% or 0.4%, of TAC postbiotic lotion were respectively applied on the 10 subjects` face on different visits and the degree of hydration was recorded at two time points, 0 and 3 h (Figure 3A and Figure 3B). The moisturizing degree at 0 h displayed no significant difference among three concentrations of TAC postbiotic lotions (Figure 3A). The 0.4% TAC postbiotic lotion showed a better moisturizing effect than 0.2% lotion, and a significant increase in the degree of hydration when comparing to the control group ($p < 0.05$) (Figure 3B). Therefore, the concentration of 0.4% TAC postbiotics was selected for the further clinic trail of a long-term treatment.

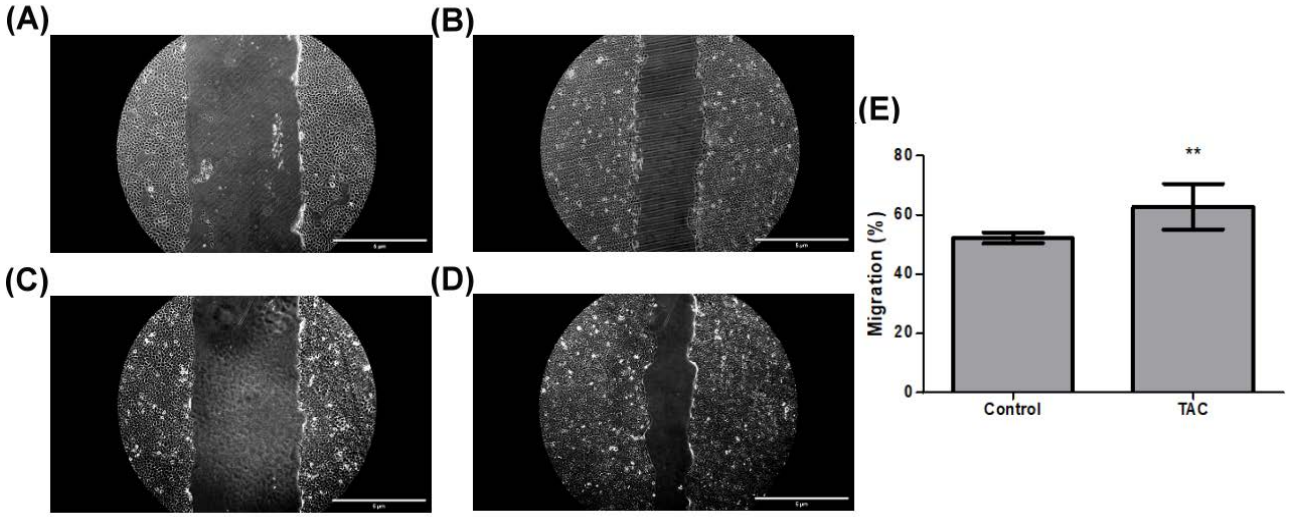

Figure 2. TAC postbiotics increases basal layer of human keratinocytes (HaCaT) (2×10^6 cells) migration. (A) Control, 0 h; (B) Control, 24 h; (C) 0.3125% TAC, 0 h; (B) 0.3125% TAC, 24 h; (E) Wound healing assay compared TAC with control in 24 h treatment. Migration distance was measured using Image J program. ** $p < 0.01$ as compared to that of the control. Scare bar is 5 μm

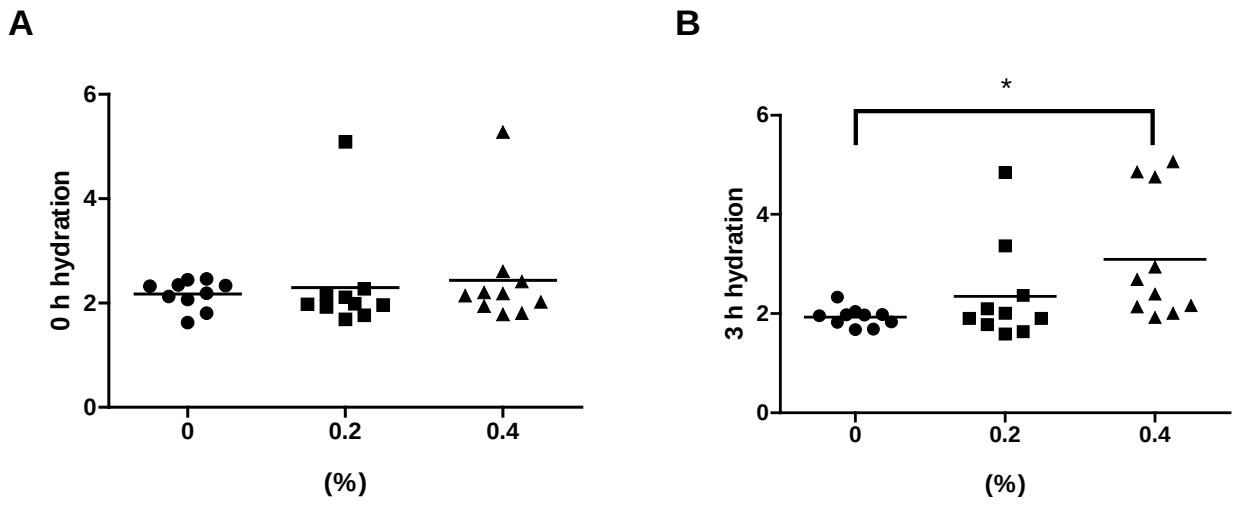

Figure 3. Comparison of the hydration degree of 0, 0.2 and 0.4 concentrations of TAC postbiotics lotion on the face of human clinical trial. (A) Apply for 0 h; (B) Apply for 3 h. n =10 in each concentrations, * $p < 0.05$ vs. 0% concentration

3.4. Effects of a Long-term Treatment of the TAC Postbiotic Lotion on Human Clinic Trail

To evaluate the effect of TAC lotion in a long-term application, twenty subjects were participated in the human clinic trail. Medical visits were scheduled on week 0, 1, 2, and 4. All subjects were submitted to a pre-test and passed the evaluation of the skin safety test (Figure S1). All participants accomplished and no withdrawal occurred before the end of the trail. The overall results of the 4-week treatment were summarized in Table 1.

3.4.1. Effects of the TAC Postbiotic Lotion on Human Skin Hydration

The level of skin hydration was improved in TAC lotion group, and showed values of 107.3, 108.3 and 108.3, on week 1, 2, and 4, respectively, when comparing to that baseline (100.0, p <0.05; 100.0, p < 0.001; 100.0, p <0.001, respectively) of each group. The level of skin hydration was also improved in placebo lotion group, and showed values of 106.5, 107.9 and 108.1, on week 1, 2, and 4, respectively, when comparing to that baseline (100.0, p < 0.05; 100.0, p < 0.05; 100.0, p < 0.01, respectively) (Figure 4A). The improvement of skin hydration was observed both in TAC and placebo groups, and the effect lasted from week 1 to 4 throughout the trail.

3.4.2. Effects of the TAC Postbiotic Lotion on Human Skin Sebum

After 4 weeks of application, no significant difference in relative sebum content was displayed between placebo and TAC lotion groups when comparing to that baseline of each group (Figure 4B). However, the relative sebum content showed a gradual reduction from 100 to 93.8, which indicated a decrease by 6.2% during the 4 weeks of TAC lotion application (Figure 4B; Table 1). In contrast, the value only decreased by 3.0% in placebo group during the 4 weeks as compared with the baseline on week 0 (Table 1).

Table 1. Summary of improvement effects of skin condition after the study (n =20; + positive improvement; - negative improvement)

Item	Ratio of improved subjects (improvement of skin parameter (%))	
	Placebo lotion	TAC lotion
Hydration	+8.1%	+8.3%
Sebum	-3.0%	-6.2%
Skin inflammation index	-7.3%	-9.2%
Brightness	+2.1%	+2.7%
Spots	-4.2%	-9.7%
Wrinkles	-10.5%	-20.7%

Note: Improvement of skin parameter (%): (the mean value at week 4- the mean value at the baseline)/ the mean value ate the baseline × 100%

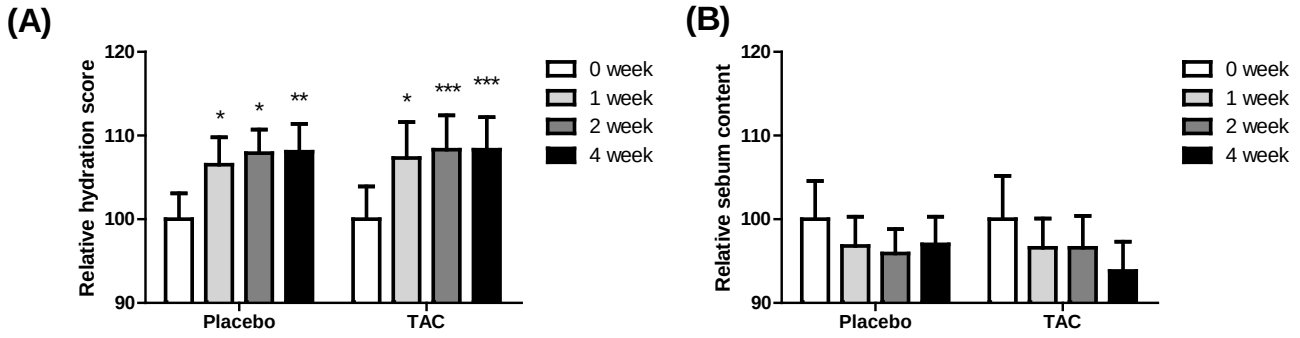

Figure 4. Improvement of hydration and sebum content for TAC and place lotion on 0, 1th, 2th and 4th weeks. (A) Hydration; (B) Sebum content. Bars represent mean ± SEM. n = 20 in each group. *** p < 0.01, ** p < 0.01,* p < 0.01 vs. 0 week with in group

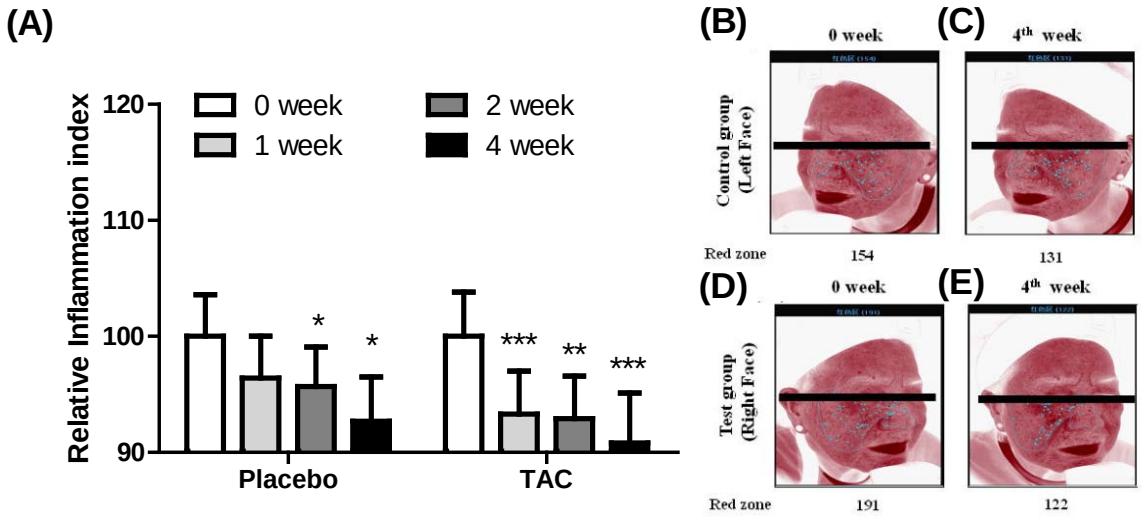

Figure 5. Improvement of inflammation for TAC and place lotion. (A) Relative inflammation index detected by chroma meter at 0, 1, 2 and 4th weeks; n = 20 in each groups. *** p < 0.01, ** p < 0.01,* p < 0.01 vs. 0 week with in group. Bars represent mean ± SEM. (B) Red zone (inflammation) detected by VISIA at 0 and 4th weeks. (a) Placebo lotion treatment on left face at 0 week. (b) Placebo lotion treatment on left face at 4th week. (c) TAC lotion treatment on right face at 0 week. (d) TAC lotion treatment on right face at 4th week. The value showed the degree of inflammation

3.4.3. Effects of the TAC Postbiotic Lotion on Human Skin Inflammation

The index of skin relative inflammation was detected by chroma meter (Figure 5A). The level of inflammation index was significantly reduced to 93.3 from 100 on week 1 as compared to baseline on week 0 (100.0, $p < 0.001$), and the improvement remained significant throughout the 4 weeks. In contrast, the improvement in the placebo lotion group was not significant on week 1, and showed significance later on week 2 (Figure 5A). The skin inflammation of the subjects in TAC group was significantly decreased by 9.7% on week 4 as compared with the value of the baseline on week 0. In contrast, the result in placebo group was only decreased by 7.3% (Table 1). Further, the inflammation of blood vessels in the deep layers of the skin was observed by VISIA microanalysis skin image analyzer. The optical red zone in deep skin was detected and indicated with pale blue. After applying with placebo lotion for 4 weeks, the number of red zone area showed only a reduction of 23 (from 154 to 131) (Figure 5B and Figure 5C). A better reduction of 69 was seen in TAC group than in placebo group, of which the number of red zone area decreased from 191 to 122 (Figure 5D and Figure 5E). Hence, TAC lotion could effectively improve skin inflammation.

3.4.4. Effects of the TAC Postbiotic Lotion on Human Skin Brightness

The level of skin brightness was evaluated by the color performance after placebo or TAC lotion treatment. The skin brightness of the subjects in TAC group was significantly increased to 100.6 and 102.7 on week 2 and 4, respectively, as compared with baseline (100.0, $p < 0.01$; 100.0, $p < 0.001$, respectively) on week 0 (Figure 6A). In contrast, the value in placebo group was only increased to 102.1 on week 4 as compared with baseline on week 0 (Figure 6A). In conclusion, the skin brightness of the subjects in TAC groups was significantly increased by 2.7% on week 4 as compared to the baseline on week 0 (Table 1).

3.4.5. Effects of the TAC Postbiotic Lotion on Human Skin Spots and Wrinkles

The results show the spots in the TAC lotion group were significantly decreased on week 2 and 4. The value of baseline was normalized to 100 by the analyzer and the value was reduced to 94.9 and 90.3, respectively, in TAC group on week 2 and 4 as compared to the baseline (100.0, $p < 0.05$; 100.0, $p < 0.01$) on week 0. In contrast, the spots in the placebo lotion group showed no difference as compared to the baseline on week 0 (Figure 6B).

After 4 weeks of application, the skin wrinkles of the subjects in TAC group were significantly decreased from 100 to 79.3 as compared with baseline (100.0, $p < 0.05$) on week 0 (Figure 6B). In contrast, the value was only decreased to 89.5 in placebo groups on week 4 and showed no significant difference as compared with baseline on week 0 (Figure 6C). In conclusion, the skin spots and wrinkle of the subjects in TAC groups were significantly decreased by 9.7% and 20.7%, respectively, on week 4 as compared to the baseline on week 0 (Table 1).

3.5. Effect of TAC Lotion on Skin Wound

Two of the subjects were atopic dermatitis (AD) patients, and came into this trial with cracked skin on hands. The effect of TAC lotion on the wound healing was observed in these two atopic dermatitis subjects after the human clinic trail. On day 0, subject-1 had a wound on the finger, and subject-2 had multiple wounds on the palm. The wound on the finger of subject-1 showed a significant improvement, especially after 7 days of TAC application (Figure 7A and Figure 7B). The multiple wounds on the palm of subject-2 also healed significantly after 7 days of TAC application (Figure 7C and Figure 7D). The result suggested that TAC lotion was applicable on AD skin without irritation, and could improve the wound healing.

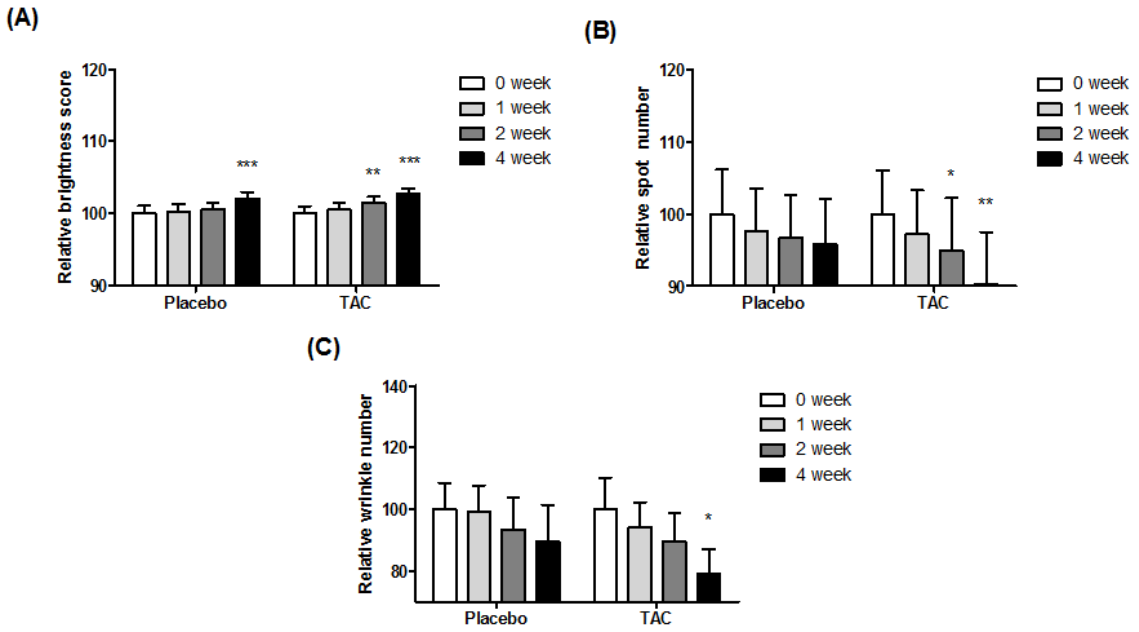

Figure 6. Improvement of skin brightness, spots and wrinkle for TAC and placebo lotion applied at 0, 1st, 2nd and 4th weeks. (A) Relative brightening score; (B) Relative skin spot numbers; (C) Relative wrinkle number. $n = 20$ in each group. *** $p < 0.01$, ** $p < 0.01$,* $p < 0.01$ vs. 0 week with in group

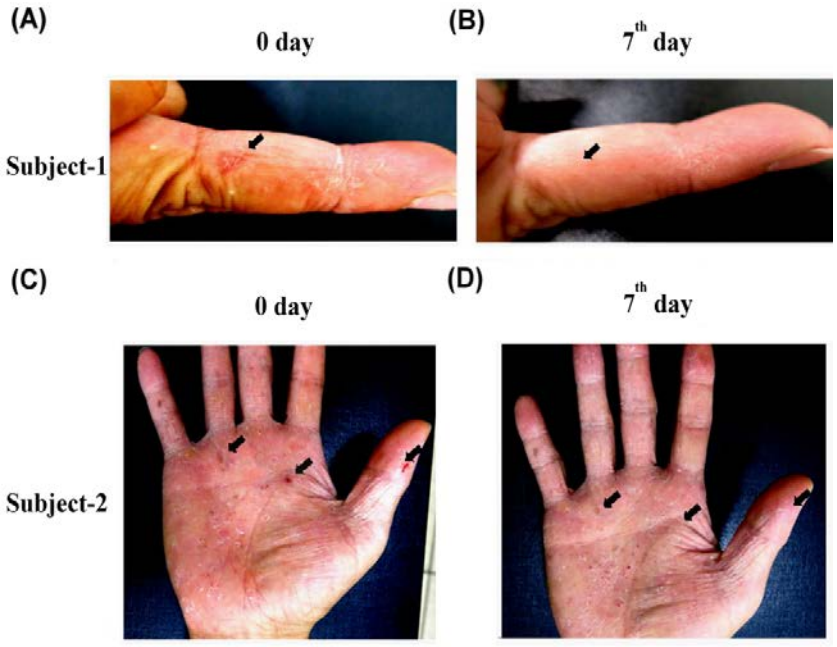

Figure 7. Improvement of atopic dermatitis skin wound repair with TAC lotion applied for 7 day. (A) Subject-1`s finger at 0 day; (B) Subject -1`s finger at 7th day; (C) Subject -2`s palm at 0 day; (D) Subject -2`s palm at 7th day. Arrow indicated the place of skin wound

4. Discussion

The antibacterial activity of probiotic bacterium is a living strategy for its own survival, and the mechanism of the competition exists diversities. TYCA06 is a strain of *Lactobacillus acidophilus*, and the antibacterial activity of this species was noted very early in 1949 [22]. AP-32 is a strain of *L. salivarius*, and a novel bacteriocin-like substance produced by this species with activity against *Enterococcus faecalis*, *E. faecium*, and *Neisseria gonorrhoeae* was characterized in 1999 [23]. CP-9 is a strain of *Bifidobacterium animalis*, and Bificin C6165 is a novel bacteriocin isolated from another strain of this species with remarkable potency for *Alicyclobacillus* control in 2013 [24]. Studies have shown that a common benign bacterium can modify its local habitat and inhibit growth of a pathogenic bacterium by releasing antibacterial free fatty acids from host skin surface triacylglycerol [25]. In other words, the antibacterial activity of probiotic bacterium was performed highly through its postbiotics, which comprise metabolites and/or cell-wall components released by probiotics [19]. In this study, the growth inhibition against the pathogenic bacteria *S. aureus* was seen in eight cell-free postbiotics, and three postbiotics of probiotic strains, TYCA06, AP-32, and CP-9, showed significantly better inhibition rates than other five.

Despite of the equivalent antibacterial activities, these three postbiotics showed different capabilities of cytokine inductions on TGF-β and IL-10 *in vitro*. TYCA06 showed a moderate induction on TGF-β levels, and the best induction on IL-10 levels among eight postbiotics. AP-32 displayed the best induction on TGF-β levels, and a moderate induction on IL-10 levels among eight postbiotics. Although CP-9 presented an antibacterial activity as strong as TYCA06 and AP-32, the inductions on TGF-β and IL-10 levels were relatively mild. Studies have shown the intervention of probiotics can modulate the immune response [26]; however, immunomodulatory effects of probiotics on pro-inflammatory and anti-inflammatory cytokine production have both been seen in different animal models [27]. The immune response toward different probiotic strains and their postbiotics cannot be interpreted easily, and sometime the result can be opposite. For instance, the amounts of secreted cytokines by dendritic cells after stimulation with cells of three trains of *L. plantarum*, Lp790, Lp813 and Lp998, resulted pro-inflammatory whereas stimulation with culture supernatants (postbiotics) increased the release of the anti-inflammatory IL-10 cytokine and inhibited the release of IL-12p70 [28]. Therefore, more investigation is needed to elucidate the role of postbiotics of TYCA06, AP-32 or CP-9 in the immunomodulatory. The aim of this study focused on screening for outstanding antibacterial activities, which can solely be exerted by the postbiotics. The postbiotics of TYCA06, AP-32 and CP-9 were combined and the effect of this postbiotic blend on skin health was evaluated by a clinical trial in human.

Healthy skin is the primary defense system of human body from pathogens, and open wound on skin can increase the opportunity for bacterial load. The facilitation of wound healing can reduce the risk of bacterial infection, and probiotics have shown such beneficial effects in burn patients [29]. Another research has shown that oral supplementation with a sterile lysate of probiotics is sufficient to boost systemic oxytocin levels and improve wound repair capacity [30]. AD is a common dermatologic disease which is frequently accompanied with inflamed and cracked skin. The human trial in this study did not set any specific criteria for enrollment, and most of our volunteers were healthy subjects. Only two subjects happened to be AD patients, and this TAC lotion improved the wound healing of their cracked skin without any irritation or side effects. Although the case number of AD was low, this preliminary result sheds some light on the topical use of postbiotics for sensitive skin. The theory behind this potential use of TAC formula was also

supported by our antibacterial activity and anti-inflammation assay in *vitro*. The present of *S. aureus* can aggravate the symptom due to abnormalities in the skin barrier of persons with AD [31]. The antibacterial activity of TAC formula is able to reduce the population of *S. aureus*, and the induction of TGF-β and IL-10 is able to ameliorate the inflammation on AD skin. Besides, TAC postbiotics increased the cell migration in HaCaT cells, and indicated a potential promotion on wound healing. Therefore, another clinical trial specifically for AD patients is worthy and necessary to validate the beneficial effect of TAC formula on AD skin.

A right balance between skin hydration (moisture) and sebum (skin surface lipids) is an indication of healthy skin and plays a central role in protecting and preserving skin integrity [32]. In modern life, people often go back and forth between air-conditioning and open area. The rapid change of temperature and humidity can cause stress on skin. The transepidermal water loss increases when staying indoor under dry air and synthesis of stratum corneum lipids increases when going outdoor under sunshine [33]. The TAC lotion helped to maintain the balance by improving the skin hydration and stabilizing the sebum content. Healthy skin is the fundamental requirement for good physical health and well external appearance. In addition to the health, the bright, spotless, and wrinkleless facial skin is a beauty goal for the majority nowadays. In the past two decades, the interest in natural remedies has increased, and so as in research on the use of natural ingredients in dermatology [34]. The TAC postbiotics contain amino acids and compounds naturally fermented by probiotic bacteria, and improved skin speckles and wrinkles in our subjects. The content of postbiotics is complex and can be varied by the procedure of collection. Studies have found the abundance of short-chain fatty acids, vitamins, phenols, egzopolisaccharides, and enzymes are usually rich in postbiotics [35]. In order to have a better understanding on the effect of TAC postbiotics, further studies on the functional ingredient of the TAC postbiotics are warranted.

Author Contributions

Ho, H.H., Chen, W.C., Huang, Y.F., Lin, J.H., Hsu, C.H., Yi, T.H. and Liang, C.H. carried out the experiments. Ho, H.H., Chen, W.C., Kuo, Y.W., Lin, J. H., Hsu, C.H., Chan, L.P. and Liang, C.H. analyzed the data. Ho, H.H., Chen, W.C., Chen, J.F., Chan, L.P. and Liang, C.H. prepared figures and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

The authors are grateful to Glac Biotech Co., Ltd. (Tainan City, Taiwan) for providing the probiotics.

References

[1] Azimi, E., Xia, J., Lerner, E.A., Peripheral mechanisms of Itch. *Curr Probl Dermatol*. 50, 18-23, 2016.

[2] Thomsen, S.F., Atopic dermatitis: natural history, diagnosis, and treatment. *ISRN Allergy*. 354250, 2014.

[3] Grey, K., Maguiness, S., Atopic dermatitis: update for pediatricians. *Pediatr Ann*. 45(8), 280-286, 2016.

[4] Goh, C.L., Wong, J.S.,Giam, Y.C., Skin colonization of *Staphylococcus aureus* in atopic dermatitis patients seen at the National Skin Centre, Singapore. *Int J Dermatol*. 36(9), 653-657, 1997.

[5] Nakatsuji, T., Chen, T.H., Two, A.M., Chun, K.A., Narala, S., Geha, R.S., Hata, T.R., Gallo, R.L., *Staphylococcus aureus* exploits epidermal barrier defects in atopic dermatitis to trigger cytokine expression. *J. Invest. Dermatol.* 136(11), 2192-2200, 2016.

[6] Baquerizo Nole, K.L., Yim, E., Keri, J.E., Probiotics and prebiotics in dermatology. *J. Am. Acad. Dermatol.* 71(4), 814-821, 2014.

[7] Lise, M., Mayer, I., Silveira, M., Use of probiotics in atopic dermatitis. *Rev. Assoc. Med. Bras*. 64(11), 997-1001, 2018.

[8] Murillo, N., Raoult, D., Skin microbiota: overview and role in the skin diseases acne vulgaris and rosacea. *Future Microbiol.* 8(2), 209-222, 2013.

[9] Zeeuwen, P.L., Kleerebezem, M., Timmerman, H.M., Schalkwijk, J., Microbiome and skin diseases. *Curr. Opin. Allergy Clin. Immunol*. 13(5), 514-520, 2013.

[10] Myles, I.A., Earland, N.J., Anderson, E.D., Moore, I.N., Kieh, M.D., Williams, K.W., Saleem, A., Fontecilla, N.M., Welch, P.A., Darnell, D.A., Barnhart, L.A., Sun, A.A., Uzel, G., Datta, S.K., First-in-human topical microbiome transplantation with *Roseomonas mucosa* for atopic dermatitis. *JCI Insight*. 3(9), e120608, 2018.

[11] Simpson, P., Good bacteria for healthy skin; Ulysses Press; Berkely, CA, USA, Chapter 6, p85-103, 2019.

[12] Hiraishi, A., Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. *Lett. Appl. Microbiol*. 15, 210-213, 1992.

[13] Stackebrandt, E., Goodfellow, M., Nucleic acid techniques in bacterial systematics. *Chichester*.; Wiley: New York, NY, USA, p115-175, 1991.

[14] Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*. 173, 697-703, 1991.

[15] Strus, M., Kucharska, A., Kukla, G., Brzychczy-Włoch, M., Maresz, K., Heczko, P.B., The in vitro activity of vaginal *Lactobacillus* with probiotic properties against Candida. *Infect. Dis. Obstet. Gynecol*. 13(2), 69-75, 2005.

[16] Liu, Y.S., Wu, J.F., Huang, C.C., Assessment of bacteriostatic activities of viable and non-viable lactic acid bacteria against methicillin-resistant *Staphylococcus aureus*. *Basic Clin. Pharmacol. Toxicol.* 125, S6, 14-15, 2019.

[17] Montanari, C., Barbieri, F., Magnani, M., Grazia, L., Gardini, F., Tabanelli, G., Phenotypic diversity of *Lactobacillus sakei* strains. *Front. Microbiol*. 9, 2003, 2018.

[18] Geoghegan, J.A., Irvine, A.D., Foster, T.J., *Staphylococcus aureus* and atopic dermatitis: A complex and evolving relationship. *Trends Microbiol*. 26(6), 484-497, 2018.

[19] Aguilar-Toalá, J., Garcia-Varela, R., Garcia, H., Mata-Haro, V., González-Córdova, A., Vallejo-Cordoba, B., Hernández-Mendoza, A., Postbiotics: An evolving term within the functional foods field. *Trends Food Sci. Technol.* 75, 105-114, 2018.

[20] Lukic, J., Chen, V., Strahinic, I., Begovic, J., Lev-Tov, H., Davis, S.C., Tomic-Canic, M., Pastar, I., Probiotics or pro-healers: the role of beneficial bacteria in tissue repair. *Wound Repair Regen*. 25(6), 912-922, 2017.

[21] Giam, Y.C., Hebert, A.A., Dizon, M.V., Van Bever, H., Tiongco-Recto, M., Kim, K.H., Soebono, H., Munasir, Z., Diana, I.A., Luk, D.C., A review on the role of moisturizers for atopic dermatitis. *Asia Pac. Allergy*. 6(2), 120-128, 2016.

[22] Vincent, J.G., Veimett, R.C., Riley, R.F., Antibacterial activity associated with *Lactobacillus acidophilus*. *J. Bacteriol.* 78(4), 477-484, 1959.

[23] Ocaña, V.S., Pesce De Ruiz Holgado, A.A., Nader-Macías, M.E., Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. *Appl. Environ. Microbiol.* 65(12), 5631-5635, 1999.

[24] Pei, J., Yuan, Y., Yue, T., Characterization of bacteriocin bificin C6165: a novel bacteriocin. *J. Appl. Microbiol.* 114(5), 1273-1284, 2013.

[25] Bomar, L., Brugger, S.D., Yost, B.H., Davies, S.S., Lemon, K.P., *Corynebacterium accolens* releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. *mBio*. 7(1), e01725-15, 2016.

[26] Levy, M., Thaiss, C.A., Zeevi, D., Dohnalová, L., Zilberman-Schapira, G., Mahdi, J.A., David, E., Savidor, A., Korem, T., Herzig, Y., Pevsner-Fischer, M., Shapiro, H., Christ, A., Harmelin, A., Halpern, Z., Latz, E., Flavell, R.A., Amit, I., Segal, E., Elinav, E., Microbiota-modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. *Cell*. 163(6). 1428-1443, 2015.

[27] Azad, M., Sarker, M., Wan, D., Immunomodulatory effects of probiotics on cytokine profiles. *BioMed Res. Int.* 2018, 8063647, 2018.

[28] Zago, M., Scaltriti, E., Bonvini, B., Fornasari, M.E., Penna, G., Massimiliano, L., Carminati, D., Rescigno, M., Giraffa, G., Genomic diversity and immunomodulatory activity of *Lactobacillus plantarum* isolated from dairy products. *Benefic. Microbes*. 8(4), 597-604, 2017.

[29] Baquerizo Nole, K.L., Yim, E., Keri, J.E., Probiotics and prebiotics in dermatology. *J. Am. Acad. Dermatol*. 1(4), 814-821, 2014.

[30] Varian, B. J., Poutahidis, T., DiBenedictis, B.T., Levkovich, T., Ibrahim, Y., Didyk, E., Shikhman, L., Cheung, H.K., Hardas, A., Ricciardi, C.E., Kolandaivelu, K., Veenema, A.H., Alm, E.J., Erdman, S.E., Microbial lysate upregulates host oxytocin. *Brain, Behav., Immun*. 61, 36-49, 2017.

[31] Nakatsuji, T., Chen, T.H., Two, A.M., Chun, K.A., Narala, S., Geha, R.S., Hata, T.R., Gallo, R.L., *Staphylococcus aureus* exploits epidermal barrier defects in atopic dermatitis to trigger cytokine expression. *J. Invest. Dermatol.* 136(11), 2192-2200, 2016.

[32] Ezerskaia, A., Pereira, S.F., Urbach, H.P., Verhagen, R., Varghese, B., Quantitative and simultaneous non-invasive measurement of skin hydration and sebum levels. *Biomed. Opt. Express*. 7(6), 2311-2320, 2016.

[33] Del Rosso, J.Q., Levin, J., The clinical relevance of maintaining the functional integrity of the stratum corneum in both healthy and disease-affected skin. *J. Clin. Aesthet. Dermatol*. 4(9), 22-42, 2011.

[34] Baumann, L., Rodriguez, D., Taylor, S.C., Wu, J., Natural considerations for skin of color. *Cutis*. 78(6 Suppl), 2-19, 2006.

[35] Varian, B.J., Poutahidis, T., DiBenedictis, B.T., Levkovich, T., Ibrahim, Y, Didyk, E., Shikhman, L., Cheung, H.K., Hardas, A., Ricciardi, C.E., Kolandaivelu, K., Veenema, A.H., Alm, E.J., Erdman, S.E., Microbial lysate upregulates host oxytocin. *Brain, Behav., Immun*. 61, 36-49, 2017.

Supplementary Results

Table S1. Placebo/TAC lotion formula

Ingredient	Percentage (%)		
TAC lactobacillus ferment (*L. acidophilus* TYCA06, *L. salivarius* AP-32, *B. animals* subsp. lactis CP-9	0	0.2	0.4
Water	75-85		
Glycerol	< 10		
Sea water	< 5		
Citric acid	< 5		
Aole extract	< 5		
Cucurbita pepo (pumpkin) seed extract	< 5		
Hexadecanoic acid	< 5		
Isononyl isononanoate	< 5		
Prunus amygdalus dulcis oil	< 5		
Shea butter	< 5		
Sodium hydroxide	< 1		
Polysorbate 20	< 1		
Essential oil	< 0.5		
Carbopol 940	< 0.5		
Xantham gum	< 0.5		
Zanthoxylum piperitum fruit extract	< 0.5		
Pulsatilla koreana extract	< 0.5		
Usnea barbata (Lichen) extract	< 0.5		
Dipropylene glycol	< 0.5		
Hydroxyacetophenone	< 0.5		
Caprylyl glycol	< 0.5		
Dipotassium glycyrrhizinate	< 0.5		
Sodium hyaluronate	< 0.5		
Allantoin	< 0.5		

Note: TAC lactobacillus ferment 0% as the placebo.

Table S2. The proportion of amino acid concentrations in TAC powder

Name	Con. (mM)	(%)
ALA	11.3782	18.37
GLU	7.8119	12.61
LEU	7.8009	12.59
SER	4.8361	7.81
LYS	4.1603	6.72
ASP	4.0543	6.00
TYR	3.3720	5.44
PRO	3.0889	4.99
ILE	2.7788	4.49
GLY	2.3842	3.85
ARG	2.1176	3.42
THR	2.0987	3.39
PHE	2.0562	3.32
CYS	1.2207	1.97
VAL	1.0793	1.74
MET	0.9868	1.59
HIS	0.7185	1.16
Total	**61.9433**	**100.00**

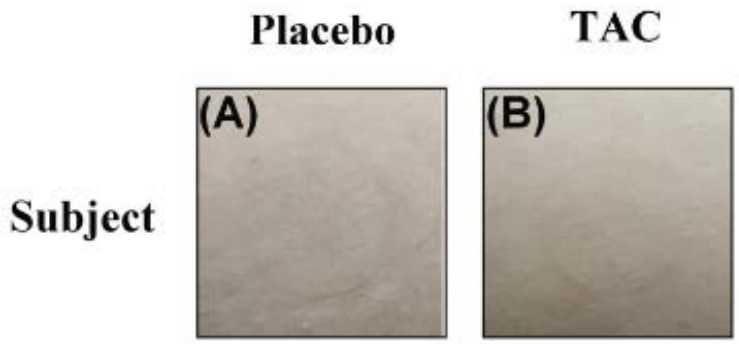

Figure S1. Skin safety test evaluation. (A) placebo, (B) TAC