Journal of Microbiology (2022) Vol. 60
DOI 10.1007/s12275-022-1520-6
eISSN 1976-3794
pISSN 1225-8873

A split face study on the effect of an anti-acne product containing fermentation products of *Enterococcus faecalis* CBT SL-5 on skin microbiome modification and acne improvement[§]

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(Received Oct 6, 2021 / Revised Jan 13, 2022 / Accepted Jan 19, 2022)

Antibiotic-resistant *Cutibacterium acnes* and dysbiosis of the skin microbiome are of increasing concern in acne treatment. *Enterococcus faecalis*, a widely used probiotic, has shown benefits for acne treatment by exerting antimicrobial activity against *C. acnes*. Therefore, this study aimed to investigate the efficacy and safety of an *E. faecalis* CBT SL-5-extract-containing lotion in patients with mild-to-moderate acne. Twenty patients were enrolled in this randomized, placebo-controlled, split-face comparative study. Patients were treated with *E. faecalis* lotion on one side of the face and a vehicle lotion on the other side for 4 weeks. The efficacy outcome measures included improvement in the investigators' assessment of acne severity, patient satisfaction, changes in skin parameters and diversity of the skin microbiome. The investigators' assessment score was significantly improved on the test side compared to the control side, after 2 weeks ($p = 0.009$) and 6 weeks ($p < 0.0005$). However, TEWL and skin hydration were not significantly different between the two groups. The phylogenetic diversity of the skin microbiota decreased over time in the skin samples of test side. In conclusion, *E. faecalis* CBT SL-5 extract can be a feasible and well-tolerated option for improving acne severity and skin microbiome dysbiosis in mild-to-moderate acne patients.

***Keywords*:** acne vulgaris, *Enterococcus faecalis*, skin microbiome, dysbiosis

Introduction

Acne vulgaris is multifactorial condition characterized by excess sebum production, follicular keratinization, colonization of pilosebaceous unit by *Cutibacterium acnes*, and inflammation. Recently, new findings regarding the role of *C. acnes* in acne was revealed: beyond its hyperproliferation, loss of balance among the different *C. acnes* phylotypes and dysbiosis of the skin microbiome may contribute to acne development (Omer *et al.*, 2017; Lee *et al.*, 2019). Furthermore, although the topical and systemic antibiotics have been one of the mainstays of treatment of acne for the past few decades, antibiotic resistance due to increasing usage of antibiotics is a major concern (Adler *et al.*, 2017; Karadag *et al.*, 2021). As a result, guidelines recommend resistance reduction strategies such as avoidance of antibiotic monotherapy, combination treatment with other topical modalities, and limited use of oral antibiotics (Adler *et al.*, 2017). In order to address these problems, there has been an increasing demand for alternatives to antibiotics. Probiotic lactic acid bacteria (LAB) have been proposed as an alternative because various scientific reports have indicated their potential for overcoming antibiotic-associated side effects (Kang *et al.*, 2009).

Enterococcus faecalis CBT SL-5, a LAB strain isolated from human feces, was reported to exert significant antimicrobial activity against Gram-positive bacteria, especially *C. acnes*, by producing bacteriocin (Kang *et al.*, 2009). Therefore, we expected that *E. faecalis* CBT SL-5 extract could be utilized for normalizing skin microbiota and clinically improving acne.

Materials and Methods

Participant

This double-blind, randomized, placebo-controlled, split-face study was approved by the Institutional Review Board of Chung-Ang University Hospital (Approval No. 1962-005-376). The study was conducted in accordance with the principles of the Declaration of Helsinki. We enrolled 20 healthy Korean volunteers (> 13 years) with mild-to-moderate acne (grade 2 to 3 on the Investigator's Global Assessment [IGA] scale) (Supplementary data Table S1). The exclusion criteria included the use of topical antibiotics or retinoids within two weeks, use of oral antibiotics within four weeks, or history of oral isotretinoin treatment within six months.

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[§]Supplemental material for this article may be found at https://doi.org/10.1007/s12275-022-1520-6.

Interventions

The study plan consisted of a screening/baseline visit (V0), follow-up after 2 and 4 weeks of treatment (V1 and V2), and a final visit (V3) at week 6, which was 2 weeks after treatment discontinuation. Each side of the face was randomly allocated to either a lotion containing *E. faecalis* CBT SL-5 extract or the vehicle lotion. The clinical team, patients, investigators, and monitors were blinded to treatment allocation. The patients were instructed to apply approximately 0.5–1 g of either test or vehicle lotion to the corresponding side of their face twice daily for 4 weeks. The composition of the *E. faecalis* CBT SL-5 extract lotion and vehicle lotion was identical, except that the vehicle lotion did not contain the *E. faecalis* CBT SL-5 extract (Supplementary data Table S2). *Enterococcus faecalis* CBT-SL5 was isolated from a healthy Korean human fecal sample. The growth condition of the strain was mentioned in the previous papers (Lee *et al.*, 2008; Kang *et al.*, 2009). Enterocin, an active ingredient which showed anti-microbial activity against *C. acnes*, was purified and verified as a bacteriocin of *E. faecalis* using N-terminal sequencing (Kang *et al.*, 2009). *Enterococcus faecalis* CBT SL-5 extract lotion was manufactured and provided by Cell Biotech, Co., Ltd. All patients were provided an identical face wash and moisturizing creams for use and prohibited from using any other cosmetic products and cosmetic procedures.

Assessment of treatment outcomes

Primary endpoint: The primary efficacy endpoint was based on the investigators' assessment of clinical improvement performed at weeks 2 (V1) and 4 (V2) after treatment and at 2 weeks after treatment discontinuation (V3). Two blinded investigators assessed clinical improvement using a 5-point modified global improvement scale (0, worse; 1, no change; 2, approximately 25% improvement; 3, approximately 50% improvement; and 4, approximately 75% improvement), modified from investigator's global evaluation of improvement and Kligman score from previous studies (Lucky *et al.*, 1998; Dréno *et al.*, 2011). Clinical photographs were taken using the JANUS® instrument (PSI Co. Ltd.) at each visit, and the investigators were provided with all of the photographs taken at each time of assessment, which allowed accurate evaluations.

Secondary endpoint: Secondary endpoints included (1) treatment success rate, defined as the percentage of patients with an IGA score of 0 or 1 at each visits, (2) subjective satisfaction score (Supplementary data Table S3), (3) changes in the biophysical parameters of skin barrier function (skin hydration and transepidermal water loss [TEWL]), and (4) changes in the skin microbiota composition. Corneometer® CM825 and Tewamater® TM300 (Courage & Khazaka GmbH) were used to assess skin hydration and TEWL. Skin surface sampling for microbiome analysis was performed on both cheeks of each patient using skin swabs (skin swab samples) and pore strip methods (sebum samples). The skin swab was performed by rubbing 10 times horizontally and 10 times vertically with a cotton swab in a 4 cm^2 area of each cheek. The pore strip method was performed by applying a pliable, adhesive tape on the nose to collect sebum samples. Before each measurement of skin biophysical parameters and skin swab sampling, patients were prohibited from using face wash and creams for 12 h and acclimatized in a room with normal temperature and humidity (22 ± 2°C and 50 ± 5% relative humidity) for 20 min. Microbiome analysis was performed using 16S rRNA sequencing results and the QIIME™ 2 (Quantitative Insights Into Microbial Ecology) pipeline 2019.7 (Bolyen *et al.*, 2019). Detailed methods related to microbiome analysis are described in Supplementary data Materials and Methods.

Safety analysis: At each visit, local and systemic adverse events (AEs) and local skin reactions (LSRs) including erythema, edema, scaling/dryness, stinging/burning, and pruritus were evaluated. Treatment compliance was evaluated at each visit using the following formula: 100 × (number of actual applications/number of scheduled applications). Noncompliance was defined as a compliance < 80%.

Statistical analyses

SPSS package (SPSS for Windows, version 24.0; SPSS Inc.) was used. Repeated measures analysis of variance (RM-ANOVA) was used for an intra-group comparison and the Chi-square test for comparing pre- and post-treatment values. A p-value < 0.05 was considered to be significant. Based on the previous research data which examined the diversity of skin micro-

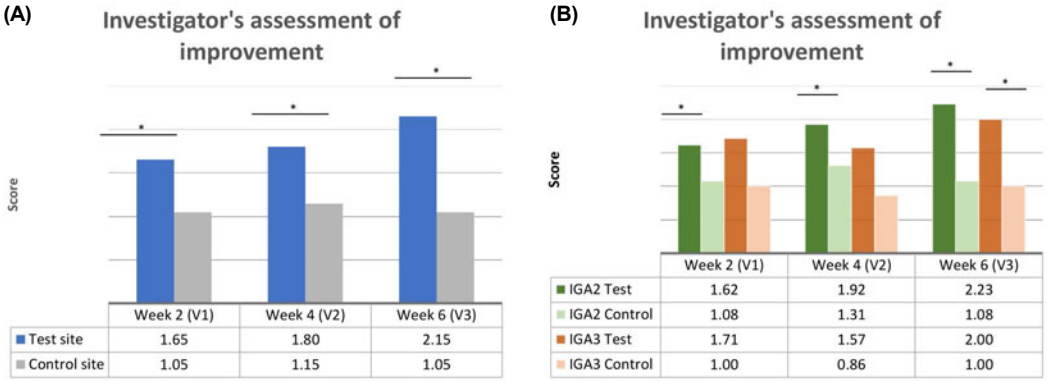

Fig. 1. Investigator-assessed clinical improvement scale. (A) Clinical assessment of improvement by independent physicians. Active group (n = 10) showed significantly higher score than placebo group (n = 10) at week 2, 4, and 6. (B) Subgroup analysis of physician's assessment score by baseline IGA (p value by RM-ANOVA test and *p < 0.05).

Table 1. Comparison of the improvement assessment score, satisfaction score, skin hydration and TEWL between two groups

			Week 0	Week 2	Week 4	Week 6
Improvement assessment score	Test group	*n*	20	20	20	20
		Mean ± SD	-	1.65 ± 0.81	1.80 ± 0.83	2.15 ± 0.88
	Control group	*n*	20	20	20	20
		Mean ± SD	-	1.05 ± 0.51	1.15 ± 0.49	1.05 ± 0.51
	p value[a]		-	0.009	0.005	0.000
Subjective satisfaction score	Test group	*n*	20	20	20	20
		Mean ± SD	-	0.55 ± 0.61	1.05 ± 0.69	1.50 ± 0.95
	Control group	*n*	20	20	20	20
		Mean ± SD	-	0.30 ± 0.57	0.75 ± 0.72	0.95 ± 0.76
	p value[a]		-	0.187	0.184	0.050
Skin hydration (A.U.)	Test group	*n*	20	20	20	20
		Mean ± SD	59.91 ± 13.46	58.60 ± 13.83	67.95 ± 11.09	65.78 ± 12.91
	Control group	*n*	20	20	20	20
		Mean ± SD	61.9 ± 14.67	57.40 ± 13.13	64.66 ± 11.54	61.75 ± 12.89
	p value[a]		0.653	0.779	0.364	0.330
TEWL (g/m^{2}h)	Test group	*n*	20	20	20	20
		Mean ± SD	18.50 ± 9.63	16.35 ± 5.45	16.89 ± 6.88	16.51 ± 9.54
	Control group	*n*	20	20	20	20
		Mean ± SD	18.95 ± 10.27	17.04 ± 6.32	17.02 ± 6.65	17.58 ± 8.53
	p value[a]		0.886	0.712	0.954	0.710

[a]*p* value by independent sample t-test and Bonferroni correction for multiple comparisons

biota after systemic antibiotic treatment, the number of patients was calculated statistically by PASS version 13.0 (NCSS Statistical Software) using one-sample paired t-test with a power of 80% and type I error of 5% (α = 0.05) (Chien *et al.*, 2019). Considering the study design of split-face study and allowing 15% extra for drop-outs, totally, 20 participants were recruited at the baseline.

Results

All 20 patients (11 men and 9 women) completed the study and all attended every follow-up visit. The patients were aged 19–38 years (26.5 ± 5.9). Thirteen patients (65%) had mild acne (IGA2) and seven patients (35%) had moderate acne (IGA3) at baseline assessed using IGA.

Investigators' assessment of clinical improvement

The mean global improvement score was significantly higher at the test side compared to control side at all time points ($p < 0.05$) (Fig. 1A, Table 1). Continuous clinical improvement was observed in the test side up to six weeks (V3). Serial JANUS® images demonstrated the improvement of acne lesions over time on the *E. faecalis* CBT SL-5-lotion-applied side (Fig. 2). Also, the overall improvement (global improvement scores) in patients with mild acne (IGA2) was faster than in patients with moderate acne (IGA3) (Fig. 1B).

Treatment success rate based on IGA scale

The treatment success rate was higher in test side (20.0%) than at the control side (5.0%) at week 4 and 6. However, statistical significant differences were not found among two groups ($p = 0.116$). Furthermore, similar to the results of investigator's assessment of clinical improvement, all of the patients who achieved treatment success were patients with mild acne (IGA2) at baseline.

Subjective satisfaction score

The mean subjective satisfaction scores were higher at the

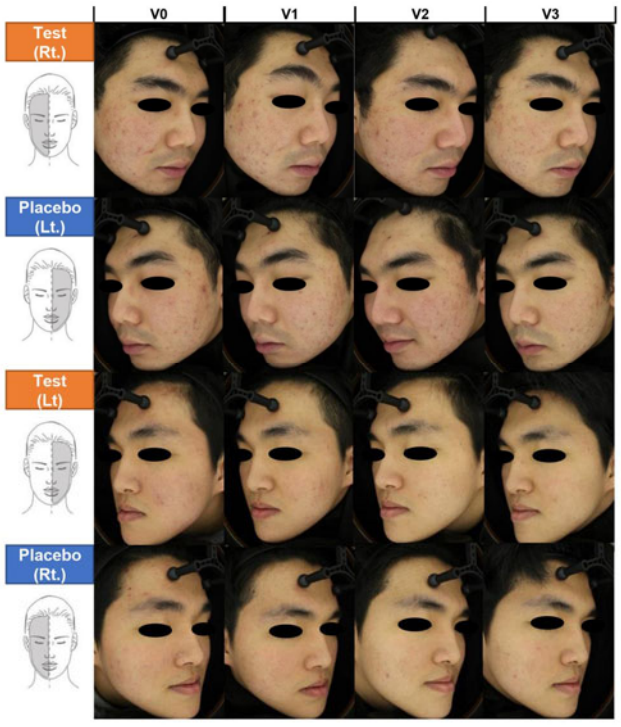

Fig. 2. Representative serial JANUS® images. Images are taken at baseline and 2, 4, and 6 weeks after the treatment.

test side than at the control side at all time points (Fig. 3A). There was a statistically significant difference at V3. Furthermore, the mean subjective satisfaction score at the test side showed a greater difference in patients with mild acne than in patients with moderate acne (Fig. 3B).

Changes in TEWL and skin hydration

In general, higher skin hydration value and lower TEWL indicates better skin barrier function. Compared to baseline, the mean TEWL decreased at all time points at both the test and control sides although the changes were not statistically significant. Although the mean TEWL at the test side was lower than that at the control side, but was not statistically significant (Fig. 3C). Furthermore, although not statistically significant, the mean TEWL was greater among patients with moderate acne than among patients with mild acne on both the test and control sides (Fig. 3D).

Similarly, skin hydration revealed increased skin hydration levels at V2 and V3 compared to baseline at both the test and control sides although not statistically significant. Furthermore, although not statistically significant, the mean skin hydration level was greater among patients with mild acne than among patients with moderate acne on both the test and control sides (Fig. 3E and F).

Changes in skin microbiota composition

Phylogenetic diversity (PD) in skin swab samples: PD, a phylogeny-based measure of intra-sample diversity (alpha di-

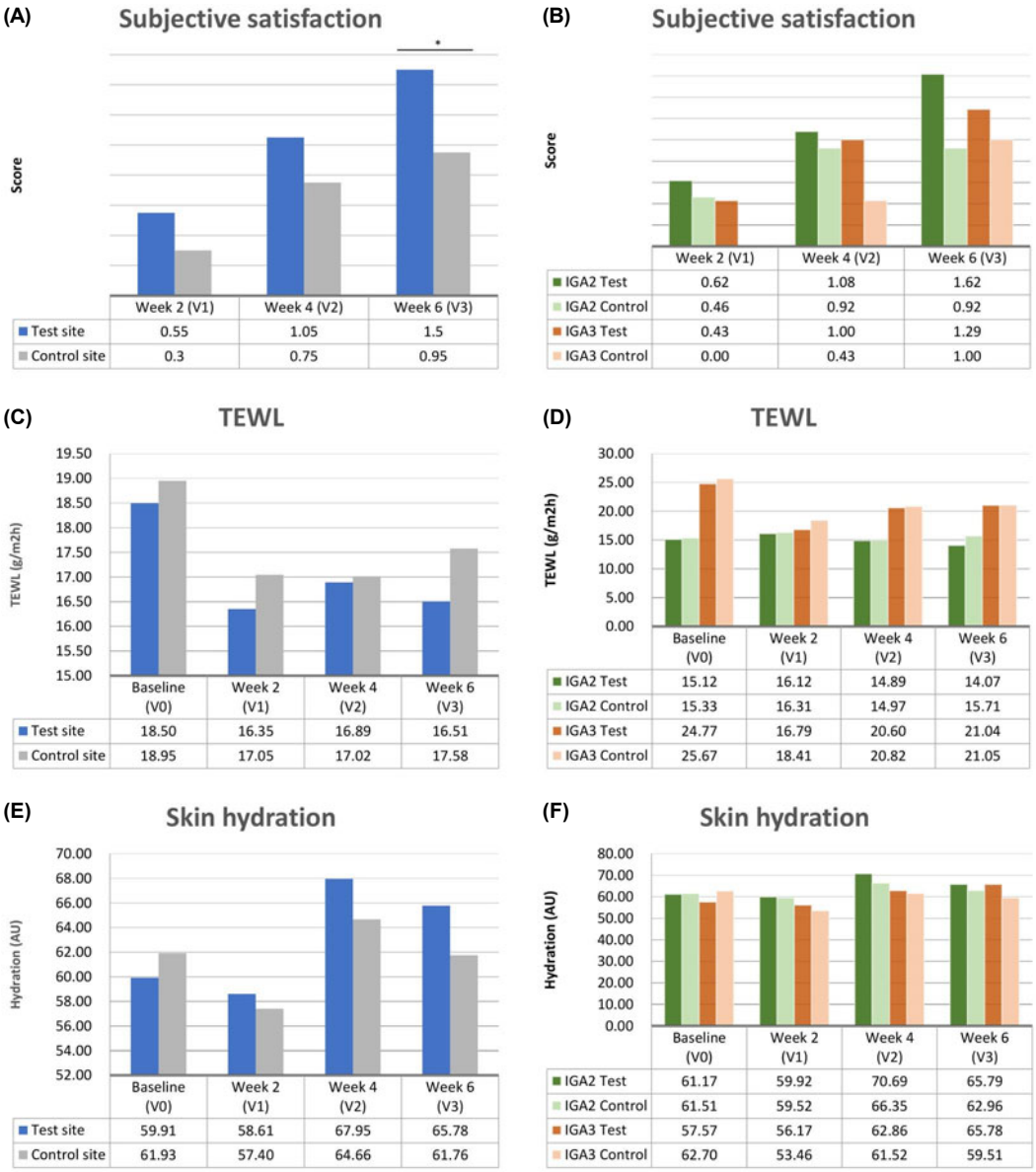

Fig. 3. Patient-assessed subjective satisfaction score, TEWL, and skin hydration. (A) Patient's satisfaction at week 2, 4, and 6, and (B) subgroup analysis of satisfaction score by baseline IGA. (C) Changes in TEWL, as measured by Tewamater®, and (D) subgroup analysis by baseline IGA. (E) Changes in skin hydration, as measured by Corneometer® CM825 at follow-up visits and (F) subgroup analysis by baseline IGA.

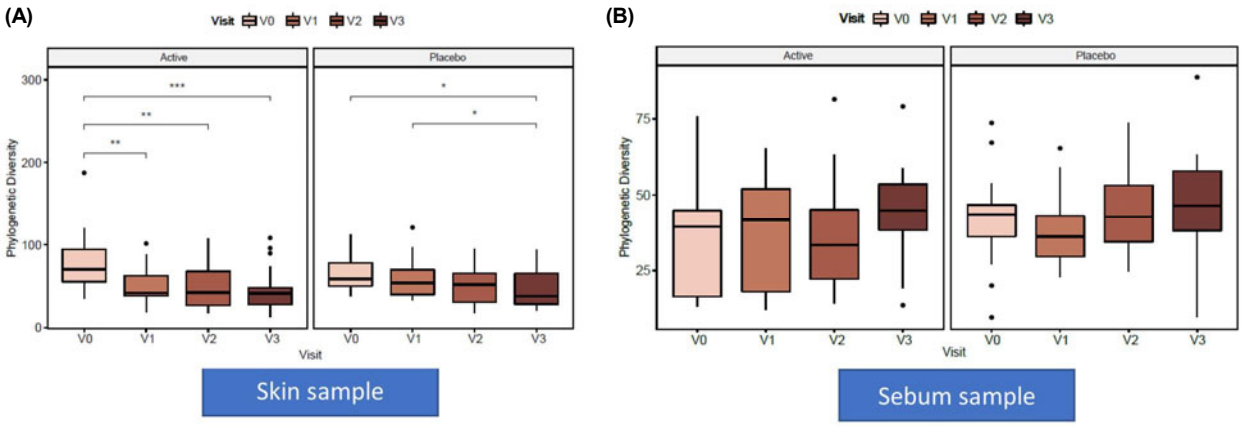

Fig. 4. Skin bacterial alpha diversity and phylogenetic diversity (PD) comparisons between active and placebo groups. (A) Comparisons of PD of all skin surface samples as time passes. Skin bacterial alpha diversity and PD comparisons between active and placebo groups for sebum extrusion samples. (B) Comparisons of PD of all sebum samples with respect to time.

versity), is calculated as the sum of the lengths of all those branches in the phylogenetic tree that span the members of clades (Armstrong *et al.*, 2021). Therefore, a low PD indicates that the species are located closer on the phylogenetic tree and are similar. To quantify the alpha microbial diversity of each sample, we used Faith's PD metric, Chao1 index, evenness, and the Shannon index. The PD of the skin swab samples from the test side significantly decreased all time points compared to that at the initial state. In contrast, at the control side, no significant decrease was observed at 2 and 4 weeks after treatment (Fig. 4A). There were no statistically significant changes in the indicators of alpha diversity other than PD, including the Chao1 index, evenness, or the Shannon index.

PD of sebum samples: The final dataset included 136 sebum samples from 17 patients because 3 of 20 patients denied sebum extrusion. Unlike the results from the skin surface swab samples, the PD did not significantly change over time at either the test or control side (Fig. 4B).

Clinical improvement in acne severity and PD in skin swab samples: We classified the test- and control-side samples as the I (Improvement) and N (Non-improvement) groups according to the investigators' assessment. At both the test and control sides, the PD was significantly lower in the I group than in the N group (Supplementary data Fig. S1A). When the samples were classified into the S (satisfied) and N (non-satisfied) groups according to the subjective satisfaction, there was no statistically significant difference between S and N at both sides (Supplementary data Fig. S1B).

In addition, the change in PD was evaluated over time between the improvement and satisfied groups (IS) *vs.* non-improvement and non-satisfied (NN) groups. For the test side samples, PD significantly decreased at V2 and V3 compared to V0 in the IS group. In contrast, in the NN group, there was no significant decrease over time (Supplementary data Fig. S1C). For the control side samples, the PD in the IS group showed a significant decrease at V3 compared to V0. However, there were no statistically significant changes in the NN group (Supplementary data Fig. S1D).

Clinical improvement in acne severity and PD in sebum samples: At the test side, PD was significantly lower in the I group than in the N group, while it was not significantly different at the control side (Supplementary data Fig. S1E). According to the subjective satisfaction score (S *vs.* N), there was no statistically significant difference between S and N nor between IS and NN at the test or control side (Supplementary data Figs. S1F–S2H).

Clinical improvement in acne severity and β diversity: We examined inter-sample diversity, or β diversity through principal coordinate analyses (PCoA) of weighted UniFrac distances. Figure 5 displays PCoA plots with inter-sample distances represented by two principal coordinates (PC2 and PC3); closely positioned samples are more similar in composition than samples at distant positions.

Clinical improvement in acne severity and β diversity in skin swab samples: The test and control sides showed no significant difference in β diversity. However, a statistically significant divergence was observed among the baseline, I, and N groups; this indicated a unique microbial signature for each group (Fig. 5A). Furthermore, upon classifying the test-side samples into the I and N group and performing LEfSe analysis, we observed that the genus level of *Cutibacterium* ASV 12210 and *Shigella* ASV 10223 and the family level of Neisseriaceae ASV 16762 was relatively higher in I than in N. In N, the genus levels of *Streptococcus* ASV 17786, *Streptococcus* ASV 4138, *Neisseria* ASV 164, *Gemella* ASV 2264, and *Sphingobacterium* ASV 17169 and family level of Burkholderiaceae ASV9855 were relatively more common than in I (data not shown).

Upon clustering the skin swab samples into the baseline, S, and N groups, we observed a significant divergence between the baseline and S groups and between the baseline and N groups (Fig. 5B).

Clinical improvement in acne severity and β diversity in skin sebum samples: Similarly, we observed a statistically significant divergence between the I and baseline groups and between the N and baseline groups (Fig. 5C). Although not statistically significant, there was a greater similarity between the I and N groups compared to that between either group

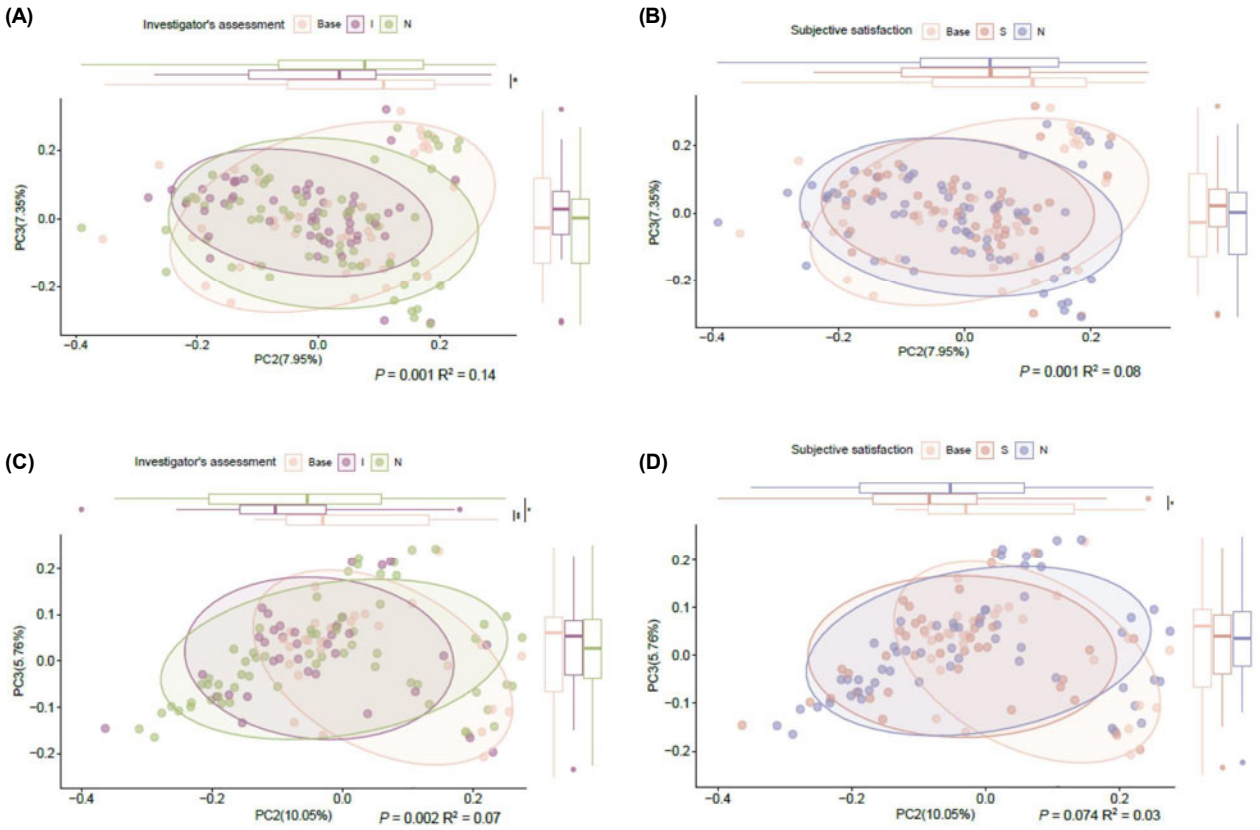

Fig. 5. Principal coordinate analysis (PCoA) of skin bacterial communities. (A and B) Principal coordinate analysis (PCoA) of skin bacterial communities based on unweighted UniFrac distance for skin swab samples. (A) Baseline (Base) *vs.* Improvement (I) *vs.* Non-Improvement (N). There was significant divergence among all groups (p = 0.001; ANOSIM). (B) Baseline (Base) *vs.* Satisfied (S) *vs.* Non-satisfied (N). There were significant differences between Base and S (p = 0.001; ANOSIM) and between Base and N (p = 0.001; ANOSIM). (C and D) Principal coordinate analysis (PCoA) of skin bacterial communities based unweighted UniFrac distance for sebum samples. (C) Base *vs.* Improvement (I) *vs.* Non-Improvement (N). There was significant divergence between Base and I (p = 0.005; ANOSIM), between I and N (p = 0.007; ANOSIM). (D) Base vs Satisfied (S) *vs.* Non-satisfied (N). There were no significant differences among all groups.

and the baseline group. Upon clustering the sebum samples into the baseline, S, and N groups, we observed no significant divergence among the groups (Fig. 5D).

Safety

Overall, the test lotion was well tolerated with similar safety profile to that of the vehicle lotion. All treatment emergent AEs (TEAEs) reported in this study (influenza A virus infection, lymphadenopathy, pelvic inflammatory disease, depression, and epigastric discomfort) were mild or moderate in severity, and none of them were related to study treatment. No LSs were observed.

Discussion

Enterococcus faecalis CBT SL-5, a strain used as a probiotic, presents both antimicrobial activity against *C. acnes* and direct anti-inflammatory activity (Lee *et al.*, 2008; Kang *et al.*, 2009). In this study, the *E. faecalis* CBT SL-5 extract-containing lotion improved acne lesions according to both investigators' assessment score and subjective satisfaction score. No significant alterations were observed on skin barrier function (TEWL and skin hydration) after using test lotion. No adverse reactions were observed, and the test lotion was well tolerated by the patients. We observed that *E. faecalis* CBT SL-5-extract-containing lotion is suitable cosmeceutical for acne patients.

According to the subgroup analysis according to baseline acne severity, faster improvement with the test lotion use was observed in the mild acne group, which suggests that this cosmeceutical may be more effective in the patients with mild acne. Similarly, all of the patients who achieved treatment success were patients with mild acne (IGA2) at baseline. This may also reflect that fact that it takes longer time to modify skin microbiome in more severe acne patients.

In addition, although more significant improvements of acne lesions were observed on the test sides of the face where *E. faecalis* CBT SL-5-extract-containing lotion was applied, some improvements were also observed on the control sides of the face as well. This may be due to the intrinsic effects of the lotion vehicle itself which contained ingredients such as hyaluronic acid and aloe vera leaf. However, more importantly, this may be due to the effects of *E. faecalis* CBT SL-5 extract lotion on the composition and diversity of the skin microbiome. Since the skin of the face is one continuous space, it

is likely that the changes in skin microbiome on one side of the face can sufficiently affect the other side of the face as well.

Regarding skin microbiome, the PD decreased over time in the skin swab samples from the test side where acne lesions improved after applying *E. faecalis* CBT SL-5 extract lotion. In contrast, several previous studies have shown an increase in α diversity after antibiotic administration, which may be due to reduced *C. acnes* colonization, which encourages the growth of other flora by freeing up niche spaces (Kelhälä *et al.*, 2018; Chien *et al.*, 2019; Lee *et al.*, 2019; Park *et al.*, 2020). This also implies increased antibiotic resistance of other species to specific antibiotics. Also, the α diversity of acne patients was reported to be greater than that of healthy individuals, suggesting that patients with acne harbor not only the proliferation of specific bacteria but also overall alteration in the skin microbiome (Li *et al.*, 2019). A low PD indicates the presence of species located closer on the phylogenetic tree and are similar with respect to ecologically preferred food, habit, and life history. The PD significantly decreased after applying the test lotion, suggesting that the bacteriocin in *E. faecalis* CBT SL-5 extract eliminates certain microbes among skin microbes, leaving only certain species located closely in the phylogenetic tree. However, since there was no change in PD in the sebum samples, the test lotion seem to not influence the skin microbiome of the infundibular population inside the skin pores. Furthermore, since those who showed objective improvement in acne (Group I) exhibited lower PD than those who had not (Group N), we observed that PD decreased as the acne improved. As mentioned above, Kang *et al.* (2009), showed that bacteriocin isolated from *E. faecalis* CBT SL-5 extract can reduce *C. acnes*. Relating to this, our results may suggest that the activity of *C. acnes* may have decrease due to changes in the interaction between skin microorganisms. Another possibility is that reduced microbial diversity resulted in decreased inflammatory responses, thus improving acne severity.

Additionally, we performed PCoA according to the results of the investigators' assessment, which indicated no significant difference in beta diversity between the groups, suggesting remarkable differences in bacterial composition between individuals (Supplementary data Fig. S2). However, according to the LEfSe results, we discovered specific ASVs in the group with improved acne; these ASVs may indicate an improvement in skin condition but further studies are needed.

Consistent with a previous report, the ratio of *Staphylococci* to *Cutibacterium* significantly increased with an increase in baseline acne severity (Supplementary data Fig. S3) (Dréno *et al.*, 2017; Kim *et al.*, 2021). Also, the mean relative abundance of both *C. acnes* and *Staphylococci* on the test side decreased after test lotion application (Supplementary data Fig. S4). A previous study has also shown that the application of the dermocosmetic reduced the number of both *Actinobacteria* and *Staphylococci* whereas topical erythromycin reduced only the number of *Actinobacteria* which reflects increased antimicrobial resistance (Chikviladze *et al.*, 2012; Juda *et al.*, 2016; Dréno *et al.*, 2017). Thus, it can be indirectly inferred that the *E. faecalis* CBT SL-5-extract-containing test lotion did not induce antibacterial resistance while reducing *C. acnes*.

This study had some limitations. The test size was small with short follow-up period, and all patients were Korean. Additionally, this study did not consider the influence of host genetics and other biological factors on individual skin microbiome diversity.

Despite these limitations, our study demonstrated the effect of *E. faecalis* CBT SL-5 extract on clinical improvement of acne as well as its effect on the composition and diversity of the skin microbiome. Based on these results, further well-designed studies with larger sample sizes and longer follow-up periods are warranted before a recommendation is made for more widespread use.

Acknowledgements

This research was supported by the Chung-Ang University Research Scholarship Grants in 2020 and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2020R1H1A1099969). The patients in this manuscript have given written informed consent to publication of their case details.

Conflict of Interest

Sanghyun Lim, Dooheon Son, and Myung Jun Chung are principal researcher, researcher, and CEO respectively at Cell Biotech, Co., Ltd.

Ethical Statements

This double-blind, randomized, placebo-controlled, split-face study was approved by the Institutional Review Board of Chung-Ang University Hospital (Approval No. 1962-005-376). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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