Efficacy and safety of human skin microbiome-derived strains as topical treatment for acne: *in vitro* and *in vivo* study

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Efficacy and safety of human skin microbiome-derived strains as topical treatment for acne: *in vitro* and *in vivo* study

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ABSTRACT

Efficacy and safety of human skin microbiome-derived strains as topical treatment for acne: *in vitro* and *in vivo* study

Acne, common among adolescents and young adults, often leads to persistent scarring like keloids and atrophic scars. The skin microbiome plays a crucial role in defending against pathogens and modulating immunity, with dysregulation linked to various skin issues, including acne. Particularly, *Cutibacterium acnes* phylotype IA-2, IB-1, and IC are associated with acne, exacerbating inflammation. In a prior study, we compiled a comprehensive skin microbiome database from healthy individuals, identifying four strains—*Staphylococcus epidermidis*(*S. epidermidis*) B424F-5, *S. epidermidis* BS47C-1, *Dermacoccus profundi*(*D. profundi*) BS35F-3, and *Streptococcus salivarius*(*S. salivarius*) BS320F-4—that exhibit antimicrobial activity against acne-causing bacteria. The effectiveness and safety of these strains on acne-related inflammation were evaluated through *in vitro* and *in vivo* experiments. *C. acnes* treatment did not exhibit cytotoxicity but increased mRNA expression of acne-related inflammatory mediators in keratinocytes without significant changes in fibroblasts. *In vivo* studies using a mouse model revealed that these strains reduced expression levels of inflammatory mediators. In conclusions, our study provides compelling evidence supporting the efficacy and safety of human skin microbiome-derived strains as a potential topical treatment for acne. By targeting both microbial colonization and inflammatory pathways, these strains offer a promising avenue for the development of novel acne therapeutics.

Key words : acne vulgaris, *Cutibacterium acnes*, inflammation, microbiome

1. INTRODUCTION

Acne is an chronic inflammatory condition involving the pilosebaceous unit that affects up to 90% of teenagers.[1] Severe form of acne, a prevalent condition during adolescence and young adulthood, is often associated with the development of recalcitrant scars such as keloids and atrophic scarring. The lingering consequences, marked by compromised skin integrity and aesthetic damage, result in long-term sequelae, imposing substantial impediments on the social, psychological, and functional aspects of patients' lives. The pathogenesis of acne involves increased sebum production, inflammatory mediators of the skin, and follicular keratinization of the pilosebaceous ducts. The bacterial colonization is known to exacerbate the pathology as a secondary factor, aggravating the diseases' severity.[2]

The human microbiome, encompassing microorganisms and genetic information constituting an ecosystem within the human body,[3] has garnered increasing attention for its intricate connection to human health. Consequently, research exploring the interplay between various diseases and the microbiome has witnessed a surge. Termed the skin microbiome, microorganisms residing on the skin act akin to their counterparts in the gut, exhibiting protective functions against pathogenic infections, participating in immune modulation, and contributing to the breakdown of substances. Dysbiosis of the microbiome (an imbalance in beneficial commensal microorganisms and pathogens) is associated with a variety of disease states on the skin.[4] Notably, in acne, a specific subgroup, such as Cutibacterium acnes phylotype IA-2, IB-1, and IC, proliferates more in individuals with severe acne than in those with normal skin, triggering inflammatory responses and correlating with increased severity of acne.[2]

In preceding studies, our research group successfully collected skin swab samples from 26 healthy individuals, constructing a comprehensive database of skin microbiome data. The isolation of over 1,630 strains of skin-derived microorganisms was achieved. Furthermore, antimicrobial activities against skin pathogens and opportunistic pathogens such as *Staphylococcal aureus*, *Bacillus subtilis*, and *Cutibacterium acnes* (*C. acnes*), were assessed based on these strains and their derived antimicrobial substance (bacteriocin). Through these experiments, we identified four skin microbiome-derived strains (*Staphylococcus epidermidis* B424F-5, *Staphylococcus epidermidis* BS47C-1, *Dermacoccus profoundi* BS35F-3, *Streptococcus salivarius* BS320F-4) exhibiting antimicrobial activity against *C. acnes*, which is known to be associated with the pathogenesis of acne.

Hence, this study aims to validate the efficacy and safety of candidate strains and their metabolites, potentially displaying therapeutic effects for acne, through preclinical models (*in vitro, in vivo*) for the validation of therapeutic candidates.

2. MATERIALS AND METHODS

2.1. *In vivo C. acnes*-induced inflammation

2.1.1. Animal treatment

Female CD-1 mice, 6 weeks old, were purchased from OrlentBio (Seongnam, Korea) and acclimated for one week. The animals were divided into five experimental groups, each containing five mice. The experimental animals were maintained under controlled conditions at a temperature of 24°C ± 0.5°C, humidity of 55%-65%, and a 12-hour light-dark cycle. They had free access to experimental animal feed. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Yonsei University (Approval No. 2023-0111) and conducted in compliance with ethical regulations in the SPF facility. After acclimation, the dorsal hair of all mice was shaved with an electric clipper to create a 2.0 x 2.0 cm area on their backs. Starting the next day, the mice received intradermal injections of approximately $1x10^7$ CFU of *C. acnes* in 20 µl of media on both sides of their backs for two weeks. Following this period, 50 µl of the homogenized supernatant from the three skin-derived strains in culture media (Tryptic soy broth for SE1 and SE2, Brain heart infusion medium for SS, and Trypticase soy yeast extract medium for DP) was applied to the skin and reapplied daily for one week. All animals were sacrificed on day 22 The degree of clinical inflammatory changes was assessed using digital photography at baseline, week 2, and week 3 after the C. acnes injection.

2.1.2. Histopathology and RT-PCR measurement of inflammatory markers

Tissue samples from mice were collected on day 22. Both sides of the animals' backs were excised, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Sections were sliced at approximately 4 µm thickness and stained with hematoxylin for examination. To assess changes in the expression of inflammatory markers, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed. The tissues were homogenized in 0.9% saline (1 ml per back biopsy) using a hand tissue grinder. The specific primer pairs used in this study are listed in **Table 1**. mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene to normalize gene expression levels.

Table 1. Sequence of primers for qRT-PCR for mouse genes

Target gene	Primer sequences (5′ → 3′)
Tnf-α	Forward: 5′- GGTGCCTATGTCTCAGCCTCTT -3′
	Reverse: 5′- GCCATAGAACTGATGAGAGGGAG -3′
Il-1β	Forward: 5′- TGGACCTTCCAGGATGAGGACA -3′
	Reverse: 5′- GTTCATCTCGGAGCCTGTAGTG -3′
Il-6	Forward: 5′- TACCACTTCACAAGTCGGAGGC -3′
	Reverse: 5′- CTGCAAGTGCATCATCGTTGTTC -3′
Il-8	Forward: 5′- GGTGATATTCGAGACCATTTACTG -3′
	Reverse: 5′- GCCAACAGTAGCCTTCACCCAT -3′
Cox2	Forward: 5′- GCGACATACTCAAGCAGGAGCA -3′
	Reverse: 5′- AGTGGTAACCGCTCAGGTGTTG -3′
inos	Forward: 5′- GAGACAGGGAAGTCTGAAGCAC -3′
	Reverse: 5′- CCAGCAGTAGTTGCTCCTCTTC -3′

Abbreviations : qRT-PCR, Quantitative reverse transcription-polymerase chain reaction; Tnf-*α*, tumor necrosis factor-alpha; *Il,* Interleukin; *Cox2,* Cyclooxygenase2; *inos,* inducible nitric oxide synthase

2.2. Cell Culture

Human dermal fibroblast (HDF) and human epidermal keratinocyte (KC) cells were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and ATCC® (Manassas, VA, USA), respectively. HDF cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin–streptomycin (Gibco). KC cells were maintained in KBM Gold Basal Medium (Lonza) with KGM Gold Single Quots supplements (Lonza). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Bacterial Culture

Reinforced Clostridial Medium (Oxoid) is used for culturing *C. acnes* ATCC 6919 under anaerobic conditions. The four strains of bacteria were named as follow: *S. epidermidis* B424F-5 (SE1), *S. epidermidis* BS47C-1 (SE2), *D. profundi* BS35F-3 (DP), *S. salivarius* BS320F-4 (SS). The four strains of bacteria (SE1, SE2, DP, SS) were cultured under aerobic condition at 37°C for 24 hours with Tryptic soy broth for SE1 and SE2, Brain heart infusion medium for SS, and Trypticase soy yeast extract medium for DP. After that, culture media were centrifuged (10,000 rpm, 5 min, 4°C) and the bacterial cells were harvested and washed twice with PBS. The optical density of the culture broth was adjusted to 2.5~3.0 at 600 nm, then centrifuged again to collect the pellet, which was resuspended in 1ml of 1×PBS. The mixture of culture supernatant and bacterial cells is then heat-treated in an 80°C water bath for 1 hour.

2.4. Cell viability Assay

The viability of keratinocytes (KC) and human dermal fibroblasts (HDF) under co-culture with C. acnes were analysed by the CCK-8 method using the cell counting kit-8 assay kit obtained from Dojindo Molecular Technologies (Japan) as the instruction of the reagent. Briefly, cells (5×10^4 cells/well) were seeded in a 96-well cell culture plate and incubated for 24 h. *C. acnes* was added at five different concentrations ($1x10^6$, $4x10^6$, $1x10^7$, $4x10^7$, $1x10^8$ CFU). Absorbance was measured at 450 nm using an ELISA microplate reader (VersaMax; Molecular Devices, California, CA, USA).

To assess the cytotoxicity of four candidate bacterial strains on KCs, WST assay (GenDepot, USA) was performed. KCs were seeded in a 96-well plate at a density of 5×10^4 cells/well and incubated for 24 hours. Subsequently, heat-treated bacterial strains were added to achieve a concentration of 1×10^7 cells, and the cells were cultured at 37°C under 5% CO_2 conditions for 16 hours.

2.5. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

To analyze the changes in mRNA expression levels for interleukin (IL)-1β, IL-6, IL-8, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF)-α in keratinocytes, qRT-PCR was performed. Total RNA was extracted using the RNAiso Plus kit (Takara Bio, Kusatsu, Shiga Prefecture, Japan) following the manufacturer's instructions and quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After cDNA synthesis using the RNA to cDNA EcoDry™ premix kit (Takara Bio, Berkeley, CA, USA), mRNA levels were measured using qRT-PCR with SYBR Green Master Mix (4309155; Promega Corporation, Madison, WI, USA) on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The specific primer pairs used in this study are listed in **Table 2**. mRNA levels were calculated using the 2-ΔΔCt method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as the housekeeping gene to normalize gene expression levels.

Table 2. Sequence of primers for qRT-PCR for human genes

Target gene	Primer sequences (5′ → 3′)
TNF-α	Forward: 5′- CTCTTCTGCCTGCTGCACTTTG -3′ Reverse: 5′- ATGGGCTACAGGCTTGTCACTC -3′
IL-1β	Forward: 5′- CCACAGACCTTCCAGGAGAATG -3′ Reverse: 5′- GTGCAGTTCAGTGATCGTACAGG -3′
IL-6	Forward: 5′- AGACAGCCACTCACCTCTTCAG -3′ Reverse: 5′- TTCTGCCAGTGCCTCTTTGCTG -3′
IL-8	Forward: 5′- GACCACACTGCGCCAACAC -3′ Reverse: 5′- CTTCTCCACAACCCTCTGCAC-3′
COX2	Forward: 5′- CGGTGAAACTCTGGCTAGACAG -3′

	Reverse: 5′- GCAAACCGTAGATGCTCAGGGA -3′
iNOS	Forward: 5′- GCTCTACACCTCCAATGTGACC -3′
	Reverse: 5′- CTGCCGAGATTTGAGCCTCATG -3′

Abbreviations : qRT-PCR, Quantitative reverse transcription-polymerase chain reaction; *TNF-α*, tumor necrosis factor-alpha; *IL,* Interleukin; *COX2,* Cyclooxygenase2; *iNOS,* inducible nitric oxide synthase

2.6. RNA sequencing (RNA-seq)

Total RNA was extracted using the RNAiso Plus reagent (Takara Bio, Kusatsu, Shiga Prefecture, Japan) following the manufacturer's instructions and dissolved in trizole solution. A library was prepared using the TruSeq RNA Library Prep Kit(Illumina, San Diego, CA, USA). The quality of RNA sequencing paired-end reads was assessed with FastQC (Illumina). RNA-Seq libraries were constructed using a SureSelect^{XT} RNA-Seq Library Prep Kit (Agilent Technologies) and sequenced in 100-bp paired-end mode on a NextSeq550-platform. Sequencing was performed by the Macrogen(Gangnam-gu, Seoul, Korea). The trimmed reads were mapped to the known reference genome using the HISAT2 program. Following read mapping, transcript assembly was performed with StringTie. This process yielded expression profile values for each sample based on known transcripts, and organized the data into read count, FPKM (Fragments Per Kilobase of transcript per Million mapped reads), and TPM (Transcripts Per Kilobase Million) metrics. Using these values, DEG (Differentially Expressed Genes) analysis was conducted with DESeq2 for four comparison sets (Acne vs. Con, Acne vs. SE2, Acne vs. SS, Acne vs. DP). From this analysis, 913 genes were identified that met the criteria of |fc| >= 2 & nbinomWaldTest raw p-value < 0.05 in at least one comparison set.

2.7. Statistical Analysis

The data are presented as mean ± standard deviation. Statistical analyses were performed using SPSS statistical software version 25.0 (IBM Corp., Armonk, NY, USA). An unpaired Student's t-test was used for comparisons between two groups, while one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was employed for comparisons among multiple groups. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Effects of the three strains on *C. acnes*-induced inflammation *in vivo*

3.1.1. Clinical photography and Histopathology

CD-1 mice were intradermally injected with a C. acnes strain at a concentration of $1x10^7$ CFU in 20 µl on both sides of the back for two weeks. Following this, the Acne group received no treatment, while the SE2, SS, and DP groups were topically treated with each respective strain at 50 µl per day for one week. The topical application was carried out by dropping the measured amount onto the induced acne lesions and gently tapping the area with a plastic spatula at least 40 times. Tissue samples were obtained on Day 22 after daily application from Day 15 to Day 21. **(Fig. 1)**

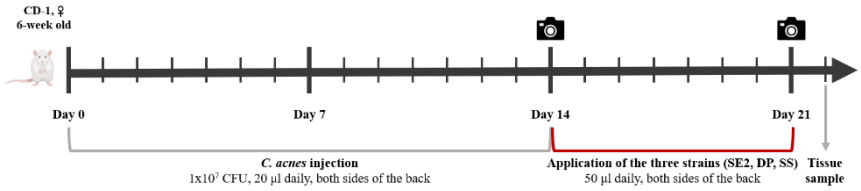

Figure 1. Schematic overview of the in vivo study. Six-week-old CD-1 female mice were injected intradermally with approximately $1x10^7$CFU of *C. acnes* in 20 µl on the back for 2-week for acne induction. After that, the homogenized supernatant of the three strains (SE2, DP, SS) in PBS media were applied for 1-week.

In **Fig. 2**, when comparing the photographs taken on day 14 to those taken on day 21, the Acne group showed a tendency for the swelling and erythema to persist. Conversely, in the SE2, SS, and DP groups, noticeable reductions in swelling were observed in the photographs taken on day 21 compared to those taken on day 14, along with a decrease in the degree of erythema.

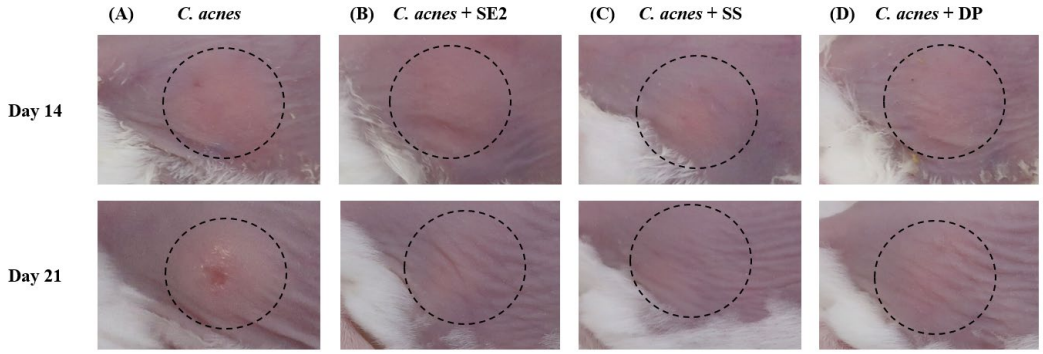

Figure 2. Representative photographs of C. acnes-induced lesions. In compare with the photographs taken on day 14 to those taken on day 21, the Acne group showed a tendency for the swelling and erythema to persist. Conversely, in the SE2, DP, and SS groups, noticeable reductions in swelling were observed in the photographs taken on day 21 compared to those taken on day 14, along with a decrease in the degree of erythema. The circular areas indicated by the dashed black lines represent the site where *C. acnes* injection and application of the three strains were conducted. *Abbreviations: SE1*, S. epidermidis B424F-5; *SE2*, S. epidermidis BS47C-1; *SS*, S. salivarius BS320F-4; *DP*, D. profoundi BS35F-3; *C. acnes*, Cutibacterium acnes.

In **Fig. 3**, it is evident that compared to the Control group, the Acne group showed mixed inflammatory cell infiltration, including lymphocytes, histiocytes, and neutrophils, extending into the dermis areas containing hair follicle units and subcutaneous fat. In contrast, SE2, SS, and DP groups, which received topical application of the respective strains for one week following *C. acnes* injection, demonstrated a reduction in the degree of inflammatory cell infiltration.

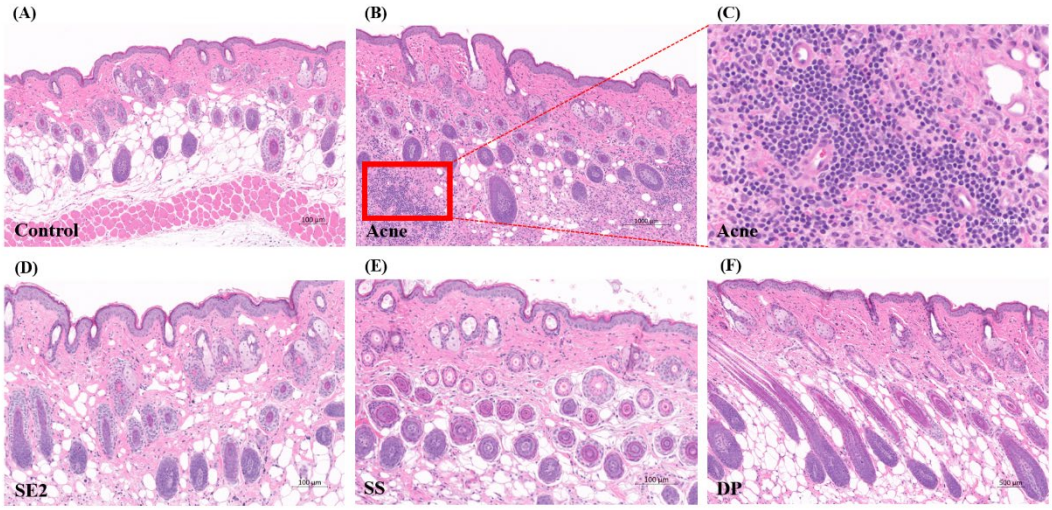

Figure 3. Histopathological evaluation of *C. acnes*-induced lesions. In compared with control group, acne group showed mixed inflammatory cell infiltration, including lymphocytes, histiocytes, and neutrophils, extending into the dermis areas containing hair follicle units and subcutaneous fat. In contrast, SE2, SS, and DP groups, which received topical application of the respective strains for one week following C. acnes injection, demonstrated a reduction in the degree of inflammatory cell infiltration.
Abbreviations: Acne, Acne group; *SE1*, *S. epidermidis* B424F-5; *SE2*, *S. epidermidis* BS47C-1; *SS*, *S. salivarius* BS320F-4; *DP*, *D. profoundi* BS35F-3

3.1.2. Realtime-PCR measurement of inflammatory markers

Injection of *C. acnes* significantly increased the expression of *Il-1β*, *Il-8*, *Tnf-alpha*, *inos*, and *Cox2* compared to the control group. However, when the bacterial strain was applied along with *C. acnes* injection, there was a significant decrease in inflammatory markers compared to the group injected with *C. acnes* alone. **(Fig. 4)** This reduction in inflammatory markers upon bacterial strain application suggests a potential therapeutic effect in acne-related inflammation.

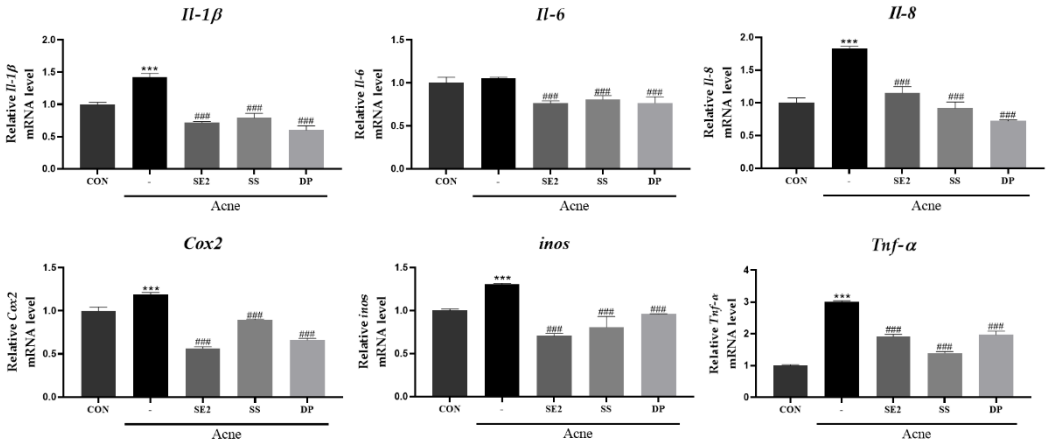

Figure 4. Realtime-PCR measurement of inflammatory markers *in vivo*. *C. acnes* injection significantly increased the expression of *Il-1β*, *Il-8*, *Tnf-alpha*, *inos*, and *Cox2* compared to the control group. There was a decrease in these inflammatory mediators when the three strains were applied along with C. acnes injection, compared to the group injected with *C. acnes* alone.
*** $p<0.005$, independent samples t-test compared with the Control group. ### $p<0.005$, independent samples *t*-test compared with the Acne group.
Abbreviations: CON, Control; -, Acne group; *SE2*, S. epidermidis BS47C-1; *SS*, S. salivarius BS320F-4; *DP*, D. profoundi BS35F-3; *Il-1β*, Interleukin-1β; *Il-6*, Interleukin-6; *Il-8*, Interleukin-8; *Cox2*, Cyclooxygenase-2; *inos*, Inducible nitric oxide synthase; *Tnf-α*, Tumor necrosis factor-α

3.2. Cell viability assay

3.2.1. Evaluation of the effect of *C. acnes* on cell cytotoxicity

To assess cytotoxicity induced by *C. acnes* treatment on keratinocyte cells and fibroblasts, a CCK-8 assay was conducted. As shown in **Fig. 5**, both KCs (A) and HDFs (B) exhibited an increase in cell viability (%) with an increase in the concentration of *C. acnes* from 10^6 CFU to 10^8 CFU. This indicates that *C. acnes* does not induce cytotoxicity on KCs and HDFs.

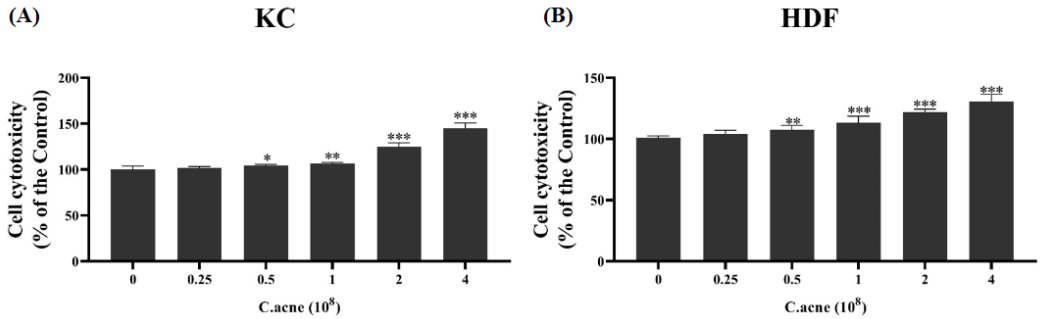

Figure 5. Cell viability after treatment of C. acnes on the keratinocytes (KCs)(A) and human dermal fibroblasts (HDFs)(B). *C. acnes* was added at five different concentrations ($1x10^6$, $4x10^6$, $1x10^7$, $4x10^7$, $1x10^8$ CFU) on KCs (A) and HDFs (B). Cell viability increased with the increase in CFU of *C. acnes*, which means there was no cytotoxicity of C. acnes on both KCs and HDFs.
(* $p < 0.5$,** $p<0.05$, *** $p < 0.005$, independent samples *t*-test compared with the Control group.)
Abbreviations : KC, Keratinocyte; *HDF*, Human dermal fibroblast; *C. acnes*, Cutibacterium acnes.

3.2.2. Evaluation of the effect of the four strains on cell cytotoxicity

To evaluate the cytotoxicity of the four strains—*S. epidermidis* BS47C-1(SE1), *S. epidermidis* B424F-5 (SE2), *S. salivarius* BS320F-4 (SS), *D. profoundi* BS35F-3 (DP)—on keratinocyte cells, a WST-1 assay was conducted. As depicted in **Fig. 6**, while the positive control group treated with DMSO showed about 5% decrease in cell viability, the groups treated with bacterial strains exhibited over 80-90% cell viability. This indicates that the topical formulations utilizing the four bacterial strains demonstrated no cytotoxicity, affirming their safety for use.

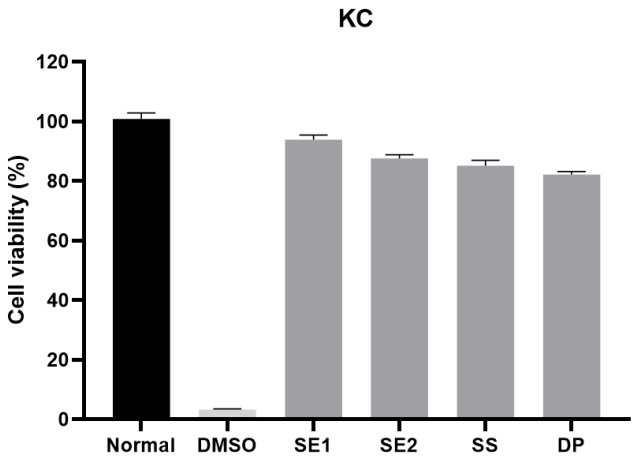

Figure 6. Cell viability after treatment of the four strains (SE1, SE2, DP, SS) on KCs. The heat-treated bacterial strains were added to achieve a concentration of 1×10^7 cells on KCs. While the positive control group treated with DMSO showed about 5% decrease in cell viability, the groups treated with bacterial strains exhibited over 80-90% cell viability.
Abbreviations : KC, Keratinocyte; *DMSO*, Dimethyl sulfoxide; *SE1*, S. epidermidis B424F-5; *SE2*, S. epidermidis BS47C-1; *SS*, S. salivarius BS320F-4; *DP*, D. profoundi BS35F-3

3.3. Evaluation of the anti-inflammatory effect of the four strains *in vitro*

To investigate alterations in the expression levels of inflammatory mediators implicated in acne pathogenesis, such as *IL-1β*. *IL-6*, *IL-8*, *TNF-α*, *iNOS* and *COX-2*, we conducted qRT-PCR after exposing keratinocyte (KC) and human dermal fibroblast (HDF) cells to *C. acnes* culture media.

The results from experiments conducted on KC cells (depicted in **Fig. 7A**) revealed a statistically significant upregulation in the expression of inflammatory mediators, including *IL-1β*. *IL-6*, *IL-8*, and *COX-2,* following treatment with *C. acnes* culture extracts compared to the control group. Conversely, qRT-PCR analysis performed on HDF cells treated with *C. acnes* culture media (depicted in **Fig. 7B**) demonstrated no statistically significant increase in the expression of inflammatory mediators relative to the control group. As a result, it can be observed that the expression of inflammatory mediators known to be associated with acne pathogenesis increases when *C. acnes* is applied to keratinocytes.

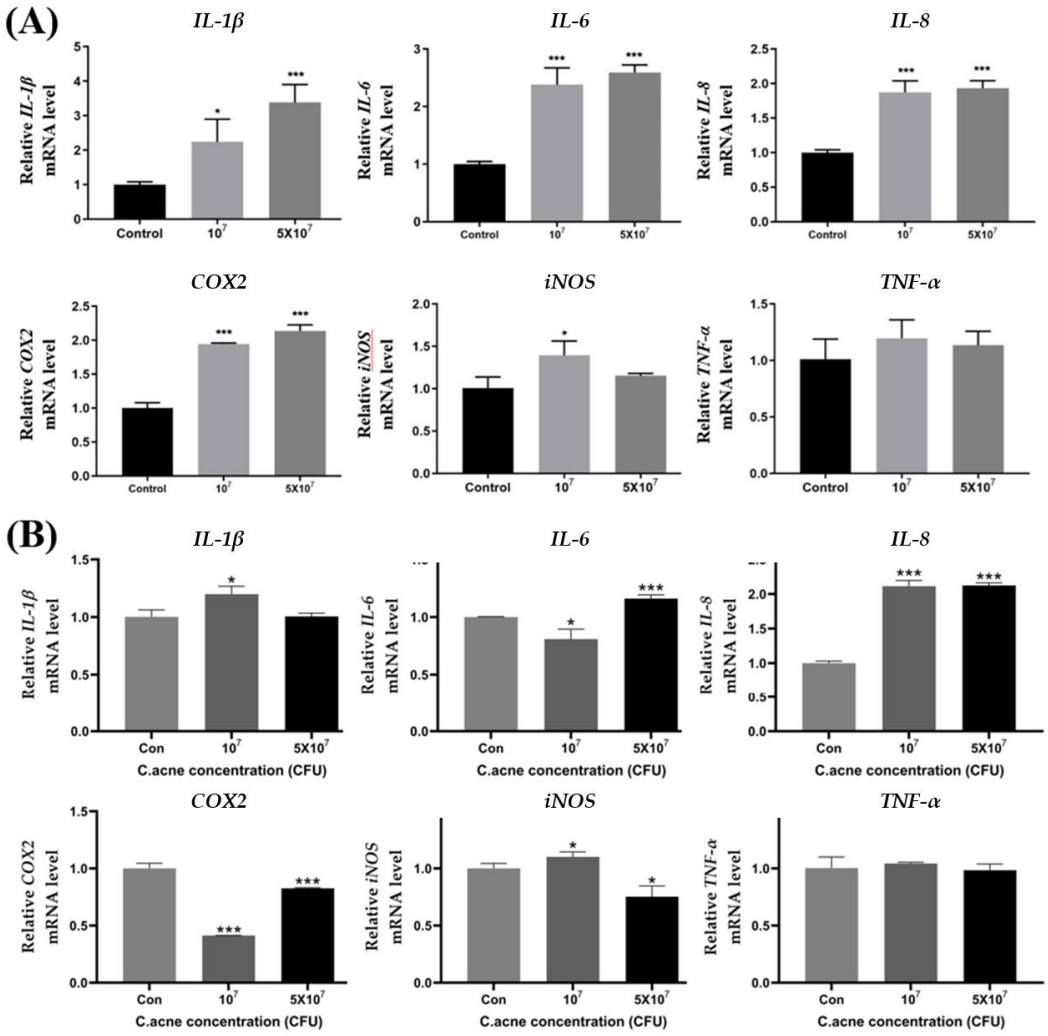

Figure 7. RT-PCR measurement of inflammatory markers in vitro after treatment of *C. acnes*.
C. acnes was treated on KCs (A) and HDFs (B) to examine the mRNA expression levels of factors associated with acne. The mRNA expression levels of most acne-related inflammatory factors (*IL-1β*. *IL-6*, *IL-8*, and *COX-2*) increased with the increase in CFU of *C. acnes* only in KC cells.
(*$p < 0.05$, ***$p < 0.005$, independent samples *t*-test compared with the Control group. The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene to normalize gene expression levels.)
Abbreviations : KC, Keratinocyte; *HDF*, Human dermal fibroblast; *C. acnes*, Cutibacterium acnes; *IL-1β*, Interleukin-1β; *IL-6*, Interleukin-6; *IL-8*, Interleukin-8; *COX2*, Cyclooxygenase-2; *iNOS*, Inducible nitric oxide synthase; *TNF-α*, Tumor necrosis factor-α

To determine whether the expression of acne-related inflammatory mediators induced by *C. acnes* could be reduced upon treatment with the four strains, qRT-PCR was conducted. In **Fig. 8** it can be observed that compared to the case where only *C. acnes* was treated in the KC cells without any intervention, treatment with the three strains, except for SE1, resulted in a statistically significant reduction in the expression of *IL-1β*, *IL-6*, *IL-8*, *COX-2*, *iNOS*, and *TNF-α*. Therefore, it can be inferred that these three strains (SE2, SS, DP) have the potential to alleviate acne-related inflammatory responses, which were subsequently utilized in the following *in vivo* experiments.

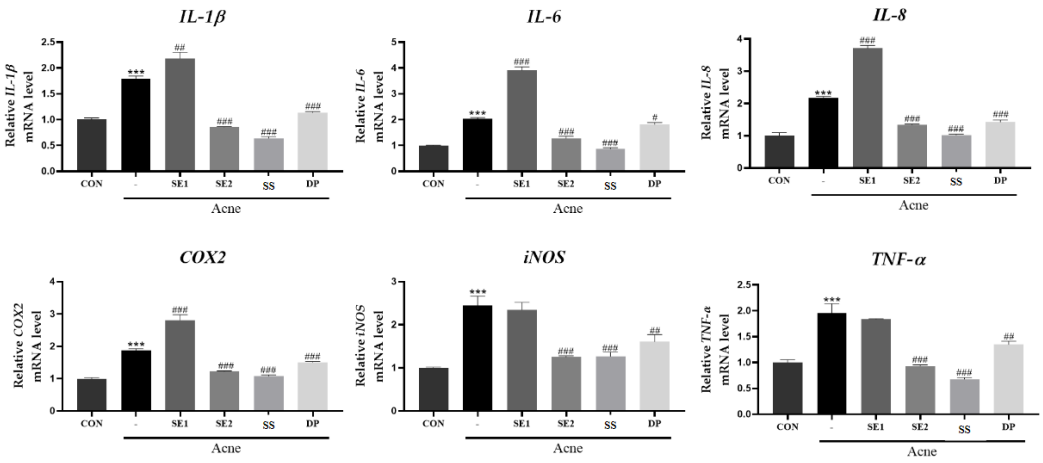

Figure 8. RT-PCR measurement of inflammatory markers *in vitro* after treatment of *C. acnes* and the four strains. *C. acnes* and the four strains (SE1, SE2, DP, SS) were treated on KC cells. In compare with the acne group, which treated only with *C. acnes*, the expression levels of mRNA of acne-related inflammatory factors (*IL-1β*. *IL-6*, *IL-8*, *TNF-α*, *iNOS* and *COX-2*) decreased for three strains (SE2, SS, DP), excluding SE1. *** $p<0.005$, independent samples t-test compared with the Control group. ### $p<0.005$, independent samples t-test compared with the Acne group.
Abbreviations: CON, Control; -, Acne group; *SE1*, S. epidermidis B424F-5; *SE2*, S. epidermidis BS47C-1; *SS*, S. salivarius BS320F-4; *DP*, D. profoundi BS35F-3; *C. acnes*, Cutibacterium acnes; *IL-1β*, Interleukin-1β; *IL-6*, Interleukin-6; *IL-8*, Interleukin-8; *COX2*, Cyclooxygenase-2; *iNOS*, Inducible nitric oxide synthase; *TNF-α*, Tumor necrosis factor-α

3.4. RNA sequencing analysis

Figure 9A and **Figure 9B** depict PCA plots and heatmaps illustrating the differences in gene expression among the Acne, SE2, SS, and DP groups. Comparing with the Acne group, significant difference in gene expression are evident in the SE2, SS, and DP groups. Furthermore, Gene Set Enrichment Analysis (GSEA) revealed that compared to the Acne group, the SE2 group exhibited increased expression of genes associated with skin barrier establishment and antimicrobial humoral response, while showing decreased expression related to cytokine stimulus and type 1/2 interferon response pathways [**Figure 9C**]. Similar changes in gene expression were observed when comparing the Acne group with the DP group [**Figure 9D**].

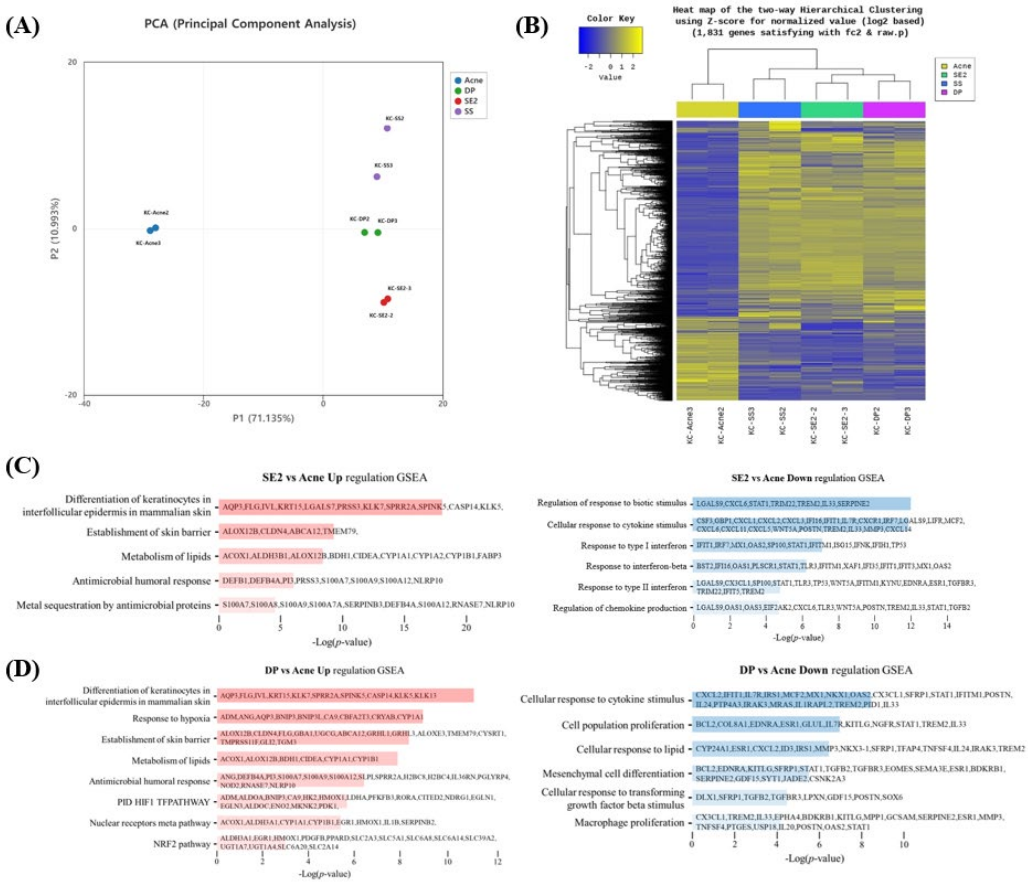

Figure 9. RNA sequencing analysis in *in vitro* samples. In (A) and (B), the PCA plot and Heatmap demonstrates distinct differences in gene expression between the Acne group and the SE2, SS, and DP groups. (C) demonstrates the result of Gene Set Enrichment Analysis (GSEA) in SE2 vs. Acne group. (D) demonstrates the result of GSEA in DP vs. Acne group. *Abbreviations : KC,* Keratinocyte; *SE2*, S. epidermidis BS47C-1; *SS*, S. salivarius BS320F-4; *DP*, D. profoundi BS35F-3

4. DISCUSSION

The primary focus in acne vulgaris treatment lies in mitigating *C. acnes*-induced inflammation, as its overgrowth is a key contributor to the condition.[6] Traditional anti-acne agents like erythromycin, clindamycin, and metronidazole have been employed for over four decades. However, their use has been limited due to potential side effects such as antibiotic resistance in *C. acnes*, erythema, scaling, dryness, burning, and pruritus.[7]

In preceding investigations conducted by our team, we isolated more than 1,630 strains of skin-derived microorganisms and subsequently evaluated their antimicrobial efficacy against opportunistic pathogens linked with acne. From this screening, we identified four skin microbiome-derived strains (*S. epidermidis* B424F-5(SE1), *S. epidermidis* BS47C-1(SE2), *S. salivarius* BS320F-4(SS), and *D. profundi* BS35F-3(예)) exhibiting promising antimicrobial activity against *C. acnes*. Our study aimed to evaluate the efficacy of these four strains in alleviating *C. acnes*-induced inflammatory responses through both *in vitro* and *in vivo* investigations. Initially, our *in vivo* study utilizing an acne animal model demonstrated that topical application of these strains led to a significant reduction in swelling and erythema compared to the control group. Histopathological analysis and qRT-PCR assessment using tissue samples provided further substantiation, revealing a decrease in inflammatory markers following treatment with the candidate strains. Additionally, we conducted cell viability tests for the four candidate strains on keratinocytes, revealing no cytotoxic effects and thereby confirming their safety profile. Furthermore, in our *in vitro* experiments, treatment with these strains resulted in a noteworthy reduction in the expression of inflammatory mediators implicated in acne pathogenesis, suggesting their potential in attenuating acne-related inflammation.

This study has confirmed the antimicrobial activity against *C. acnes* and its ability to alleviate inflammation induced by *C. acnes*. According to current research on the pathogenesis of acne, it is recognized that an increase in virulent strains of *C. acnes*, rather than a mere rise in the number of *C. acnes* organisms, is crucial in provoking the chronic inflammatory response of acne, particularly in the context of dysbiosis within the skin microbiome.[8] Notably, prevalent strains such as CC18 and ST3 clones of *C. acnes*, exhibiting virulence traits and antibiotic resistance, have been consistently identified in individuals with acne.[9] The secretion of virulence factors by *C. acnes*, encompassing lipases, proteases, hyaluronate lyase, endoglycoceramidases, neuraminidases, Christie–Atkins–Munch–Petersen (CAMP) factors, and low-molecular-weight chemotactic factors, contributes significantly to host tissue degradation and inflammation. For example, lipases serve as chemoattractants for neutrophils and catalyze the hydrolysis of sebum triglycerides, generating pro-inflammatory free fatty acids and promoting keratosis.[10] [11] Proteases and hyaluronate lyase potentially facilitate the invasion of *C. acnes* by degrading critical constituents of the extracellular matrix.[12,13] Similarly, endoglycoceramidases and neuraminidases exhibit analogous degradative activities. The resultant breakdown of the extracellular matrix enables the infiltration of inflammatory cells such as dendritic cells, leukomonocytes, neutrophils, and monocytes into the follicular wall, fostering inflammation extension into the dermis.[14] Additionally, Streptococcus-produced CAMP factors have been documented to possess pore-forming toxicity.[15] [16] Moreover,

Denda et al. elucidated that elevated CAMP levels in keratinocytes induce calcium influx, thereby impeding the recovery of epidermal barrier function subsequent to skin barrier disruption.[17 18]

This study demonstrates that heat-treated, inactivated strains derived from the skin microbiome effectively reduce inflammation associated with C. acnes-induced acne. This indicates that lipids, which are less susceptible to denaturation by heat, may play a crucial role instead of antimicrobial peptides. Fatty acids and monoglycerides, recognized as antimicrobial lipids, are known to destabilize bacterial cell membranes through several mechanisms, including (1) disrupting the electron transport chain, (2) uncoupling oxidative phosphorylation, (3) increasing membrane permeability and leakage, and (4) disrupting membrane enzyme activity. Nakatsuji's research highlights that lauric acid, a prominent antimicrobial lipid, exhibits antimicrobial activity against C. acnes. Consequently, further investigation is warranted to understand the mechanism of action of the candidate strains identified in our study.

The utilization of microbiome-based approaches in acne treatment has garnered considerable attention in recent years. Numerous studies have explored the potential of probiotics in modifying the pathophysiological factors underlying acne. Probiotics have demonstrated direct inhibitory effects on C. acnes through the secretion of antimicrobial proteins. For instance, *Streptococcus salivarius* has been shown to suppress C. acnes growth by secreting bacteriocin-like inhibitory substances.[19] Similarly, strains of *Lactococcus sp.* HY449 have exhibited the ability to block C. acnes through the release of bacteriocins.[20] Moreover, when topically applied, probiotics have been found to enhance the skin barrier function and increase antimicrobial peptide production. Notably, *Streptococcus thermophiles* has been observed to enhance ceramide production, thereby improving the skin barrier function and exerting antimicrobial effects against C. acnes.[21-23] Furthermore, probiotics exhibit immunomodulatory properties on keratinocytes and epithelial cells. For instance, *S. salivarius* strain K12 has been shown to inhibit the release of the pro-inflammatory cytokine IL-8 from keratinocytes,[24] while *Lactobacillus paracasei* NCC2461 has been found to suppress substance P-induced skin inflammation.[25 26] Collectively, these findings underscore the importance of the microbiota in acne pathogenesis and highlight the potential for modulating the microbiome for clinical improvement. The RNA sequencing results using *in vitro* samples in this study revealed that compared to the Acne group, the SE2 and DP groups showed an increase in gene expression associated with skin barrier establishment and antimicrobial humoral response, while exhibiting decreased expression related to cytokine stimulus and type 1/2 interferon response pathways as indicated by GSEA. Therefore, in addition to previous studies, our findings contributes to this growing body of knowledge by suggesting the potential therapeutic effects of acne treatment from the perspective of reducing inflammation and mitigating skin microbiome dysbiosis through treatment with skin microbiome-derived strains.

5. CONCLUSION

Overall, our study provides evidence supporting the efficacy and safety of human skin microbiome-derived strains as a potential topical treatment for acne. By targeting both microbial colonization and inflammatory pathways, these strains offer a promising avenue for the development of novel acne therapeutics. Further research, including clinical trials, is warranted to validate these findings and assess the translational potential of our experimental results in clinical settings.

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Abstract in Korean

피부 마이크로바이옴 유래 균주를 이용한 여드름 국소 치료제 개발을 위한 연구

여드름은 사회적 활동량이 가장 높은 시기인 청소년기 및 젊은 성인에 호발하며, 치료 중 켈로이드, 비후성 반흔과 같은 난치성 흉터가 발생할 수 있어 환자의 정신, 사회적 활동에 심각한 지장을 초래할 수 있는 질환이다. 휴먼 마이크로바이옴은 인간의 몸에 서식하는 미생물과 유전정보를 포함하는 생태계를 통칭한다. 이 중 피부에 존재하는 스킨 마이크로바이옴은 병원체 감염에 대한 보호작용 및 면역에 관여하고 여러 물질을 분해하는 역할을 한다고 알려져 있으며, 이러한 방어장벽이 무너지거나 균형이 파괴되면 피부 질병을 유발할 수 있다고 알려져 있다. 특히 여드름에서는 *Cutibacterium acnes*(*C. acnes*) phylotype IA-2, IB-1, IC가 정상인의 피부에서보다 여드름 환자의 피부에서 많이 발견되며, 염증성 반응을 유도하고 여드름의 중증도 증가와 연관이 있다고 보고된 바가 있다.

본 연구의 선행연구에서는 정상인의 피부 시료를 수집하여 피부 마이크로바이옴 데이터베이스를 구축하고 피부 유래 균주를 분리하였다. 해당 균주에 대해 피부 상재균 및 외인성 병원균에 대한 항균활성을 평가한 결과 여드름 병인과 관련이 있다고 알려진 C. acnes에 항균 활성을 가진 피부 유래 균주 4종을 확보하였다. (*Staphylococcus epidermidis*(*S. epidermidis*) BS47C-1, B424F-5, *Dermacoccus profundi*(*D. profundi*) BS35F-3, and *Streptococcus salivarius*(S. salivarius) BS320F-4)

본 연구에서는 *in vitro* 및 *in vivo* 실험을 통하여 해당 균주의 여드름 관련 염증반응 감소에 대한 유효성 및 안전성을 평가하고자 하였다. 피부 각질세포 및 진피 섬유아세포에 해당 균주를 처리한 결과 세포독성이 없음을 확인하였으며, 피부 각질세포에서 *C. acnes*에 의해 유도된 여드름 관련 염증반응 물질 발현 증가가 해당 균주 처리 시 감소함을 확인하였다. Mouse를 이용한 여드름 동물 모델 연구에서도 해당 균주 도포에 의하여 염증반응 물질 발현이 감소됨을 확인하였다. 따라서 본 연구 결과, 피부 마이크로바이옴 유래 균주를 이용한 항균 활성 및 항염증 효과를 기대할 수 있는 새로운 여드름 치료 도포제 개발로 이어질 수 있을 것으로 기대한다.

핵심되는 말 : 여드름, *Cutibacterium acnes*, 염증, 피부 마이크로바이옴