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Inferences in microbial structural signatures of acne microbiome and mycobiome[§]

Jubin Kim[1†], Taehun Park[2†], Hye-Jin Kim[1], Susun An[2*], and Woo Jun Sul[1*]

[1]Department of Systems Biotechnology, Chung-Ang University, Anseong 17546, Republic of Korea
[2]Safety Research Team, Amorepacific R&D Center, Yongin 17074, Republic of Korea

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Acne vulgaris, commonly known as acne, is the most common skin disorder and a multifactorial disease of the sebaceous gland. Although the pathophysiology of acne is still unclear, bacterial and fungal factors are known to be involved in. This study aimed to investigate whether the microbiomes and mycobiomes of acne patients are distinct from those of healthy subjects and to identify the structural signatures of microbiomes related to acne vulgaris. A total of 33 Korean female subjects were recruited (Acne group, n = 17; Healthy group, n = 16), and microbiome samples were collected swabbing the forehead and right cheek. To characterize the fungal and bacterial communities, 16S rRNA V4–V5 and ITS1 region, respectively, were sequenced and analysed using Qiime2. There were no significant differences in alpha and beta diversities of microbiomes between the Acne and Healthy groups. In comparison with the ratio of *Cutibacterium* to *Staphylococcus*, the acne patients had higher abundance of *Staphylococcus* compared to *Cutibacterium* than the healthy individuals. In network analysis with the dominant microorganism amplicon sequence variants (ASV) (*Cutibacterium*, *Staphylococcus, Malassezia globosa*, and *Malassezia restricta*) *Cutibacterium acnes* was identified to have hostile interactions with *Staphylococcus* and *Malassezia globosa.* Accordingly, this results suggest an insight into the differences in the skin microbiome and mycobiome between acne patients and healthy controls and provide possible microorganism candidates that modulate the microbiomes associated to acne vulgaris.

***Keywords*:** acne, skin, microbiome, mycobiome, *Staphylococcus*, 16S rRNA gene sequencing, ITS1 region sequencing

Introduction

The skin, the largest organ of the human body, is colonized by various microorganisms, and the composition of the skin microbiome includes bacteria, fungi, archaea, and viruses (Byrd *et al*., 2018). Most of the skin bacteria fall into the following four phyla: Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes (Chen and Tsao, 2013). The proportions of bacteria on the skin vary among individuals and the regions of the body. *Cutibacterium*, *Staphylococcus*, and *Corynebacterium* are the dominant genera in the facial skin and *Malassezia* is the most abundant organism in the fungal microbiome, called mycobiome, of the skin. These resident microorganisms protect the body from pathogens (Sanford and Gallo, 2013). When the normal flora is disrupted and the host immune system is weakened, opportunistic microorganisms trigger certain skin diseases (Belkaid and Segre, 2014). Several studies have shown that dysbiosis in the skin microbiome is associated with various skin diseases, such as acne vulgaris, atopic dermatitis, psoriasis, and rosacea (Fitz-Gibbon *et al*., 2013).

Acne vulgaris is one of the most common chronic skin diseases. It is a disorder by inflammation of the pilosebaceous unit and affecting approximately 85% of adolescents and young adults (Xu and Li, 2019). The clinical features of acne are comedones, called noninflammatory lesions, and papules, pustules, nodules, and cysts, called inflammatory lesions. Although the pathogenesis of acne is unclear, it is known that multi factors are involved. An increase in sebum production, follicular hyperkeratinization, and colony formation of *Cutibacterium acnes* are implicated in the pathogenesis (Bhambri *et al*., 2009). Although, recent observations suggest that the composition and activity of the skin mirobiome are related to acne development (O'Neill and Gallo, 2018), very few studies have studied the variations in the microbiome between acne patients and healthy individuals.

To investigate the differences in the skin microbiome and mycobiome between acne patients and healthy controls, we characterized the bacterial and fungal communities of the right cheek and forehead in these individuals and identified the microbiomes' structural signatures related to acne vulgaris.

Materials and Methods

Subject recruitment and sample collection

In total, 33 Korean women were recruited (17 acne patients and 16 healthy individuals) and were aged 19–28 years and had been living in Seoul, Korea, for more than three years

[†]These authors contributed equally to this work.
*For correspondence. (W.J. Sul) E-mail: sulwj@cau.ac.kr / (S. An) E-mail: ssan@amorepacific.com
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prior to the study. In order to classify the subjects, acne symptoms were evaluated according to Global Acne Severity scale (GEA scale) by dermatologist (Dreno *et al.*, 2017). Criteria for classification were: (i) Healthy group as a control included subjects with grade 0 in GEA scale, (ii) Acne group included subjects with grade ≥ 2 in GEA scale. In addition, the face was divided into six areas (forehead, cheek, nose, chin, chest, and back) and the number of acne lesions was counted at each site. All procedures were performed in a temperature and humidity controlled room and samplings were proceeded with 4 cm^2 area of right cheek and forehead by using sterile cotton- tipped swabs (COPAN Ref.165KS01), 0.15 M sodium chloride (NaCl), 0.1% Tween 20. Collected swabs were stored at -80°C until extracting the genomic DNA.

Measurement of skin parameters

After swabbing skin, all subjects washed their faces and stayed in a temperature and humidity controlled room for 30 min. We evaluated the skin conditions, such as hydration, transepidermal water loss (TEWL), sebum, and pH on the forehead and cheek. Hydration was measured using the Corneometer® CM 825 (Courage + Khazaka Electronic GmbH) and TEWL was measured with Vapometer® (Delfin Technologies). Sebum was measured with Sebumeter® SM 810 (Courage + Khazaka Electronic GmbH). Surface pH was measured using a Skin pH-meter® PH905 (Courage + Khazaka Electronic GmbH). In addition, photos of the main acne lesion were taken in order to confirm the classification of acne.

Bacterial and fungal genomic DNA (gDNA) extraction

Bacterial and fungal gDNA extraction was conducted using the PureLink® Genomic DNA Mini Kit (Invitrogen). Briefly, 400 μl of lysis buffer that contained 20 mg/ml lysozyme was added to the swab. After incubating for 1 h at 37°C, 45 μl of proteinase K was added, and then 445 μl of genomic lysis/binding buffer was added. Bead-beating was performed using a Bead Beater 16 device (Bio Spec Products Inc.) for 1 min with two 5-mm stainless beads (QIAGEN GmbH) in each tube. The tubes were incubated on ice and at room temperature for 10 min and then at 55°C for 30 min. Finally, after washing the gDNA, it was eluted with 30 μl of elution buffer and stored at -20°C until sequencing. The concentration of the gDNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.).

Sequencing of the 16S rRNA V4–V5 and internal transcribed spacer (ITS) 1 regions

The V4–V5 regions of the 16S rRNA genes of all the bacterial samples were amplified via PCR using 518F (5′-CCAGCAGCYGCGGTAAN-3′) and 926R (5′-CCGTCAATTCNTTTRAGT-3′) primers. The fungal ITS1 region was amplified using 18S-F (5′-GTAAAAGTCGTAACAAGGTTTC-3′) and 5.8S-1R (5′-GTTCAAAGAYTCGATTCAC-3′) primers. The amplification conditions were as follows: initial denaturation at 95°C for 3 min; subsequently, 25 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 5 min for the bacterial communities, and 33 cycles for the fungal communities. An index PCR was performed under the same conditions except that the amplification was performed for eight cycles. The final products were sequenced using paired-end sequencing (2 × 300 bp) on the Illumina MiSeq platform. The Illumina sequencing data were uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database. The SRA and BioProject accession numbers are SRR12968645–SRR12968710 and PRJNA669317 for the microbiome data, and SRR12968973–SRR12969038 and PRJNA673754 for the mycobiome data.

Analysis of the bacterial and fungal communities

The raw sequences were processed using Qiime2 (Quantitative Insights Into Microbial Ecology)-2019.4 (Caporaso *et al.*, 2010). The primer sequences used for the bacterial communities were removed using Cutadapt (Martin, 2011). The trimmed sequences were merged and quality-filtered using Q-score 30 (Bokulich *et al.*, 2013). The sequences were denoised using Deblur with a length of 284 nucleotides (Amir *et al.*, 2017). The bacterial taxonomy was assigned 99% by using the Greengenes database and classify-sklearn program, and the ASVs classified as mitochondria or chloroplast were eliminated. Alignment was performed using the phylogeny align-to-tree-mafft-fasttree pipeline of Qiime2, and alpha diversity (Shannon index, Faith's phylogenetic diversity [PD]) and beta diversity (Bray-curtis index, Unweighted and Weighted Unifrac Distances) were determined with a rarefied depth of 1,008 reads.

Fungal sequences (ITS1 regions) were merged and trimmed using the ITSxpress program (Rivers *et al.*, 2018). The trimmed sequences were denoised with a length of 210 nucleotides. The fungal taxonomy assignment was performed based on the UNITE database (2019.02) (Nilsson *et al.*, 2019). Alignment was performed as described above for the bacterial communities, and the sequences were rarefied with a depth of 1,892 reads.

We analyzed the bacterial communities of the Acne group by using 30 swab samples (cheek, n = 13; forehead, n = 17), and those of the healthy group by using 29 samples (cheek, n = 15; forehead, n = 14). For the fungal communities, we analyzed 32 samples from the Acne group (cheek, n = 16; forehead, n = 16) and 31 samples from the Healthy group (cheek, n = 16; forehead, n = 15). The workflow of the project was available from Figshare: https://doi.org/10.6084/m9.figshare.13498299.v1.

Table 1. The characteristics of the subjects associated with skin conditions

a. Subject information		Overall	Acne group	Normal group
Subject number		33	17	16
Age (Avg. [SD])		21.76 (2.33)	21.47 (2.40)	22.1 (2.29)
b. Skin parameters (Avg. [SD])		*p*-value		
Moisture	Cheek	*0.3131*	61.57 (5.76)	59.19 (7.82)
	Forehead	*0.5889*	61.09 (7.44)	62.38 (6.80)
TEWL	Cheek	*0.0806*	25.43 (5.68)	22.03 (5.00)
	Forehead	*0.16*	23.72 (5.53)	20.50 (3.32)
pH	Cheek	*0.6266*	6.01 (0.26)	5.99 (0.31)
	Forehead	*0.9139*	5.83 (0.59)	5.88 (0.60)
Sebum	Cheek	*0.0192**	57.06 (25.2)	38.50 (16.4)
	Forehead	*0.0279**	73.12 (25.6)	54.09 (19.1)

* $p < 0.05$, Wilcoxon-Mann-Whitney test

Microbial network analysis

To characterize the cheek and forehead microbial networks of the acne patients and healthy individuals, SParse InversE Covariance estimation for Ecological Association Inference (SPIEC-EASI) package was used in R (Kurtz *et al.*, 2015). Samples with zero abundance in each group were excluded, and bacterial and fungal ASVs with frequencies above 70% were used. For the cheek, 104 bacterial and 42 fungal ASVs were used, while 91 bacterial and 31 fungal ASVs were used for the forehead microbial network analysis.

Results

Acne patients had more sebum on their cheek and forehead than healthy individuals

To determine what skin conditions are associated with the skin microbiome in acne patients, we measured the following skin's four physiological parameters: moisture, TEWL, pH, and sebum (Table 1). Among these parameters, only sebum was significantly different in the Acne group from the Healthy group (cheek, $P < 0.05$; forehead, $P < 0.05$; Wilcoxon-Mann-Whitney test), whereas there were no significant differences between the Acne and Healthy groups in terms of moisture (cheek, $P = 0.313$; forehead, $P = 0.589$; Wilcoxon-Mann-Whitney test), TEWL (cheek, $P = 0.081$; forehead, $P = 0.160$; Wilcoxon-Mann-Whitney test), or pH (cheek, $P = 0.627$; forehead, $P = 0.914$; Wilcoxon-Mann-Whitney test) (Supplementary data Table S1).

There's no differences in alpha and beta diversity between Acne and Healthy groups

We obtained 3,201,836 merged sequence reads and 2,172 ASVs from 16S rRNA gene V4-V5 region based amplicon sequencing for the bacterial community analysis, 1,345,972 merged sequence reads and 354 ASVs from ITS1 region amplicons for the fungal community analysis.

When alpha diversity measured by the Shannon diversity and Faith's PD indices, there were no significant difference in Shannon index (Wilcoxon-Mann-Whitney test, cheek, $P = 0.061$, $P = 0.241$; forehead, $P = 0.779$, $P = 0.833$) and Faith's PD index (Wilcoxon-Mann-Whitney test, cheek, $P = 0.536$, $P = 0.732$; forehead, $P = 0.948$, $P = 0.740$) for both the bacterial and fungal communities between the Acne and Healthy groups (Fig. 1A and B, and Supplementary data Table S1). Principal Coordinates Analysis (PCoA) with Bray-curtis dissimilarity showed that the Acne and Healthy groups were not significantly different in the cheek or forehead microbiomes or mycobiomes (ANOSIM, cheek, $P = 0.569$, $P = 0.803$; forehead, $P = 0.566$, $P = 0.150$) (Fig. 1C and D). Additionally, beta diversity analysis with Unweighted and Weighted UniFrac distances revealed no significant difference between the two groups (Supplementary data Fig. S1). Notably, these data indicated no significant differences in microbiomes and mycobiomes of the Acne and Healthy groups' cheek or forehead skin surface.

Taxonomic signatures of acne in bacterial and fungal ASVs

We identified 35 bacterial phyla with 525 genera and six fungal phyla with 62 genera. In all the samples, the phylum level assignment showed that Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes were the major phyla in both the cheek and forehead microbiomes, and the Basidiomycota phylum accounted for > 98% of the fungal communities in the cheek or forehead mycobiome (Supplementary data Fig. S2). At the genus level, *Cutibacterium* and *Staphylococcus* were the most abundant bacteria in the cheek and forehead, and *Malassezia* was dominant in the fungal communities (Fig. 2A and B). Overall, these data indicated that the microbial communities' taxonomic compositions on the cheek and forehead were similar between acne patients and healthy individuals.

Even though there were no differences in overall microbiome's and mycobiome's structures, the Linear discriminant analysis (LDA) Effect Size (LEfSe) showed that some

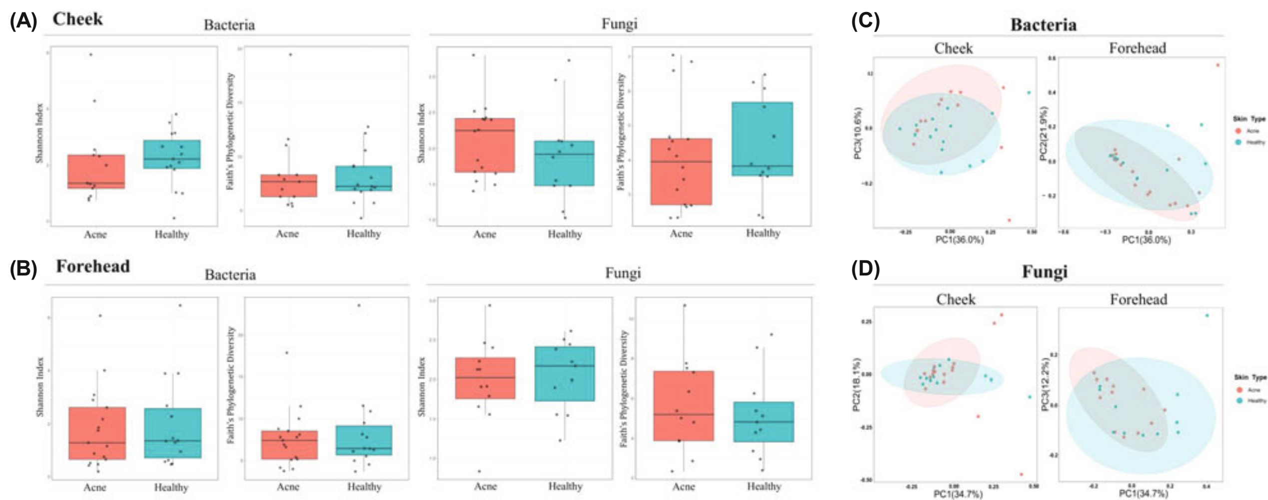

Fig. 1. Bacterial and fungal communities of the cheek and forehead microbiomes. Alpha diversities of the bacterial and fungal communities on the (A) cheek and (B) forehead, based on Shannon index and Faith's Phylogenetic Diversity (PD). Principal coordinate analysis (PCoA) of the (C) bacterial and (D) fungal communities in the cheek and forehead.

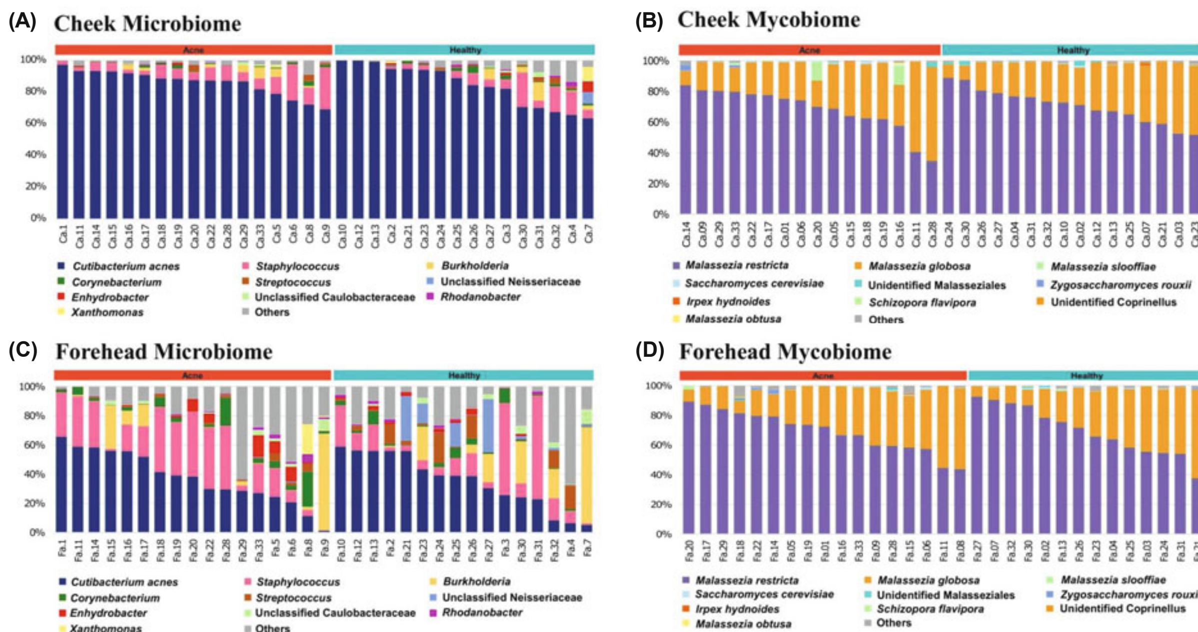

Fig. 2. Taxonomic compositions of the bacterial and fungal communities at the genus level. The relative abundance of the top 11 bacteria and fungi in the (A) cheek and (B) forehead microbiomes of the Acne and Healthy groups.

ASVs had relatively different abundance between the Acne and Healthy groups. In the cheek microbiome, 4 and 12 bacterial ASVs were identified as more abundant by LEfSe analysis in the Acne and Healthy groups. The LEfSe analysis showed that *Corynebacterium* (ASV456), *Stenotrophomonas* (ASV24), Bacteria (ASV759), and *Aeromonadaceae* (ASV1831) were more abundant in the Acne group, whereas *Moryella* (ASV34), *Actinomyces* (ASV2145), *Leptotrichia* (ASV160), Stramenopiles (ASV1587), *Dietzia* (ASV16), Ellin6513 (ASV-540), *Porphyromonas* (ASV147), *Prevotella* (ASV88), *Corynebacterium durum* (ASV1664), *Escherichia coli* (ASV764), *Actinomyces* (ASV1129), and *Finegoldia* (ASV141) were do-

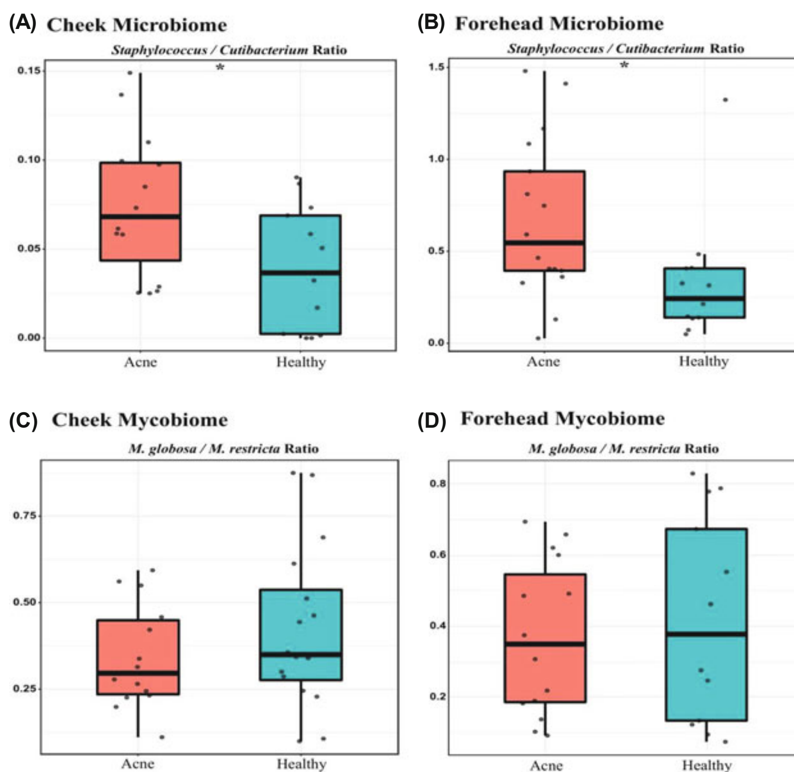

Fig. 3. The ratio of *Cutibacterium* to *Staphylococcus* and *Malassezia restricta* to *Malassezia globosa*. The ratio of the relative abundance of *Cutibacterium* to that of *Staphylococcus* in the (A) cheek and (B) forehead microbiomes after removing the outliers. The ratio of the relative abundance of *M. restricta* to that of *M. globosa* in the (C) cheek and (D) forehead mycobiomes. The statistical significance of the differences between the two groups is indicated by $^{*}p < 0.05$.

minant in the Healthy group. For fungal communities, there were seven ASVs showed in different abundances in the Acne and Healthy groups, respectively. *Malassezia globosa* (ASV211 and ASV310) and *Densospora* (ASV236) were dominant in the Acne group, whereas *Malassezia slooffiae* (ASV61), Malasseziales (ASV227), *Saccharomyces cerevisiae* (ASV22), and *M. restricta* (ASV317) were more abundant in the Healthy group.

In the forehead microbiome, two bacterial ASVs (ASV-147, *Porphyromonas*; and ASV499, *Corynebacterium*) were found only in the healthy group. In fungal ASVs, 2 and 3 ASVs were identified in the Acne (ASV10 and ASV7, *M. globosa*) and Healthy groups (ASV138 and ASV350, *M. globosa*; ASV-191, *Schizopora flavipora*), respectively. Among relatively low abundance genera, *Schizopora flavipora* (ASV191) was more abundant in the Healthy group (Supplementary data Fig. S3).

Acne had a higher ratio of *Staphylococcus* than Healthy individuals

In order to identify microbiome's structural signatures in the Acne patients group, we compared the ratios of the relative abundance of each genus. We focused on a ratio of the relative abundance of *Cutibacteirum* and that of *Staphylococcus*, as well as a ratio of the relative abundance of *M. restricta* was divided by that of *M. globosa*. The ratio of *Cutibacterium* to *Staphylococcus* was significantly higher in both the cheek and forehead microbiomes of the Acne group than in those of the Healthy group (cheek, $P = 0.048$; forehead, $P = 0.015$; Wilcoxon-Mann-Whitney test) (Fig. 3A and B). This observation indicated that acne patients had higher relative abundance of *Staphylococcus* comparing to *Cutibacterium* than healthy individuals. For the fungal communities, the ratio of *M. restricta* to *M. globosa* in the cheek or forehead mycobiome was not significantly different between the two groups (cheek, $P = 0.313$; forehead, $P = 0.751$; Wilcoxon-Mann-Whitney test) (Fig. 3C and D).

Negative interactions of microbial network analysis in Acne and Healthy group

The combined ASV data on the bacterial and fungal relative abundance were used to determine the bacteria-bacteria and bacteria-fungi interactions in the cheek or forehead in each group. For the microbial network analysis, 146 and 122 ASVs on the cheek and forehead were selected and used for network analysis. Edge density (D) corresponds to the ratio of the node connections, and transitivity (T) is the ratio between the observed number and the maximum possible number of closed triplets. The D and T values were similar between the Acne group (cheek, $D = 0.020$, $T = 0.190$; forehead, $D =$

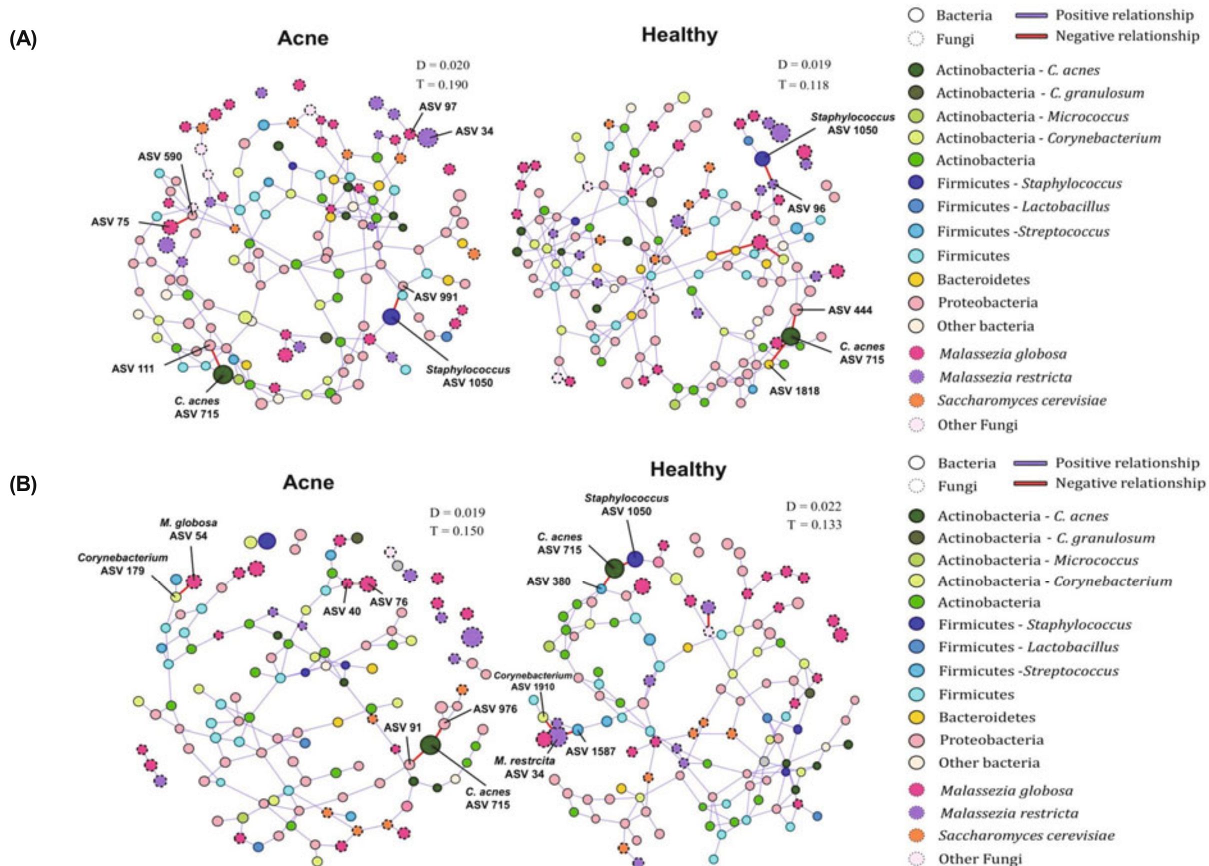

Fig. 4. Network analysis of the combined bacterial and fungal ASVs with 70% frequency. Microbial networks of the (A) cheek and (B) forehead microbiomes of the Acne and Healthy groups. Edge color indicates the correlation of two connected ASVs. Red and purple colors represent negative and positive relationships, respectively. D, density of edges; T, transitivity of the network.

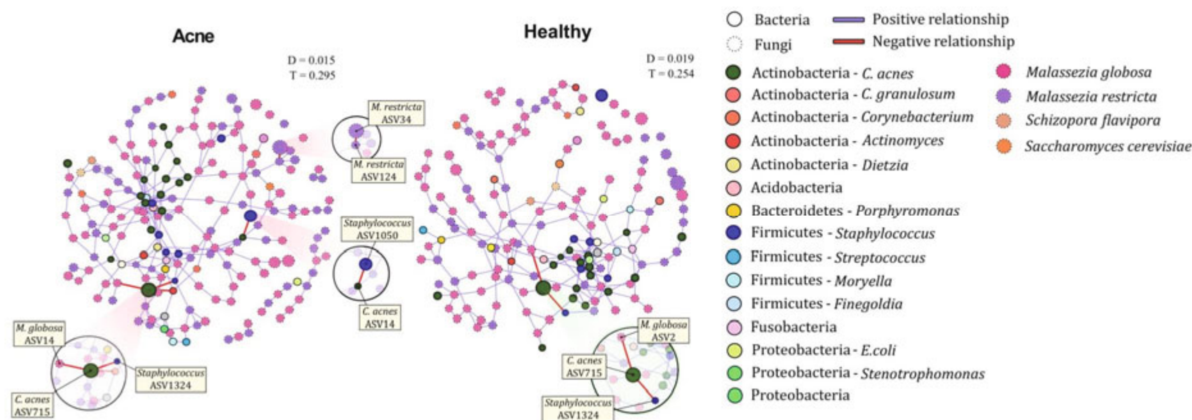

Fig. 5. Network analysis of combined bacterial and fungal ASVs based on 70% frequency in the (A) cheek and (B) forehead microbiome. Edge color indicates the relationships of two connected ASVs and each node represents an ASV.

0.019, T = 0.150) and Healthy group (cheek, D = 0.019, T = 0.118; forehead, D = 0.022, T = 0.133).

On the cheek, *Cutibacterium acnes* (ASV715) had negative interactions with *Rhodanobacter* (ASV111) in the Acne group but with *Burkholderia* (ASV444) and *Parabacteroidetes* (ASV-1818) in the Healthy group. ASV1050 affiliated to *Staphylococcus* was in hostile relations with the bacterial ASVs, such as Peptostreptococcaceae (ASV991), in the Acne group but with the fungal ASVs, such as *M. restricta* (ASV96), in the Healthy group. Besides, unlike the Healthy group, the Acne group showed negative interactions with *M. globosa* (ASV97) and *M. restricta* (ASV34). These data implicated that *M. restricta* had the negative relations among *M. globosa* in the Acne group, whereas *M. restricta* had negative interactions with *Staphylococcus* in the healthy group (Fig. 4A).

On the forehead, *C. acnes* (ASV715) negatively interacted with the bacterial ASVs of the Proteobacteria phylum (ASV-976 and ASV91) in the Acne group but with *Staphylococcus* (ASV1050) and *Streptococcus* (ASV380) in the Healthy group. Interestingly, different species of *Malassezia* had correlated with *Corynebacterium* and other bacterial ASVs. *M. globosa* (ASV54) had negative relations with *Corynebacterium* (ASV-179) in the Acne group, whereas *M. restricta* (ASV34) was in negative relations with *Corynebacterium* (ASV1910) and *Streptococcus* (ASV1587) in the Healthy group (Fig. 4B). In fungal ASVs, *M. globosa* and *M. restricta* appeared to play key roles in the Acne and Healthy groups, respectively. Thus the forehead network analysis revealed that *Corynebacterium* ASVs had negative relations with *M. globosa* ASV in the Acne group but with *M. restricta* ASV in the Healthy group. Both on the cheek and forehead, *Cutibacterium acnes* (ASV715) had negative interactions with only bacterial ASVs.

Furthermore, we collected ASVs affiliated to following four genera and species and performed network analysis; *Cutibacterium, Staphylococcus, M. globosa*, and *M. restricta*. The selected total ASVs of the Acne group were 191 (41 bacterial ASVs and 150 fungal ASVs), and of the Healthy group were 159 (40 bacterial ASVs and 119 fungal ASVs). Through this network analysis, edge density and transitivity of total ASVs were similar between the Acne and Healthy groups. Notably, *C. acnes* (ASV715) had negative relations with *Staphylococcus* (ASV1324) and *M. globosa* in both the Acne and Healthy groups. In the network of the acne group, *Staphylococcus* (ASV1050) and *C. acnes* (ASV925) had negative relations and also, between *M. restricta* (ASV34 and ASV124) had negative interactions (Fig. 5). Through network analysis using the main microorganism ASVs, *C. acnes* was identified to have negative interactions with *Staphylococcus* and *M. globosa*.

Discussion

To our knowledge, this is the first study to explore the differences in the facial microbiomes/mycobiomes between acne patients and healthy individuals via 16S rRNA gene and ITS1 region sequencing. We found that the alpha and beta diversities of the skin microbiome and mycobiome were not statistically different between acne patients and healthy individuals, but the ratio of *Cutibacterium* to *Staphylococcus* was higher in acne patients than healthy individuals.

The present study confirmed that *Staphylococcus* sp. were more abundant on the surfaces of acne lesions than on the lesion-free region of the skin in an acne patient (Dreno *et al.*, 2017), and Barnard *et al.* (2016) has described that the Propionibacteriaceae family is significantly overrepresented on the faces of healthy individuals compared with acne patients. Similar to previous findings, although the dominant bacteria in the acne patients and healthy controls were found to be *Cutibacterium* and *Staphylococcus* and the predominant fungi were *Malassezia*, we observed that the ratio of *Cutibacterium* to *Staphylococcus* was higher in the Acne group. Moreover, Park *et al.* (2017) had observed that *Staphylococcus* were associated with scalp disease and *Propionibacterium* (now *Cutibacterium*) were associated with normal scalp. This observation indicates the difference in microorganisms can lead to microbiome's dysbiosis.

In a study in which the cheek, back, and armpit microbiomes were analyzed via 16S rRNA gene sequencing, no differences were detected between acne patients and healthy controls upon alpha diversity and non-metric multidimensional scaling analyses (Kelhälä *et al.*, 2018). Accordingly, our results are in line with these results-we observed no significant dif-

ference between the acne and healthy groups. The present study described that the loss in bacterial diversity on the skins of acne patients could be related to the severity, not location, of the acne (Dagnelie *et al.*, 2019). This observation suggests that metagenomic sequencing followed by the comparison of the microbiome at the strain and genomic levels can provide more accurate information than that obtained via Illumina MiSeq. For skin sampling, method using swab does not consider the population of specific *C. acnes* on skin (Alexeyev *et al.*, 2012). However, similar to other studies that samples were taken follicles using skin biopsies or scrape, our study had large populations of *Cutibacterium* and *Staphylococcus* (Grice *et al.*, 2008; Akaza *et al.*, 2016). Although the analysis of the skin surface and follicles were similar, our study identified the differences through the skin surface analysis.

Through microbial network analysis of cheek and forehead, *M. restricta* and *Corynebacterium* play a key role in cheek and forehead microbiome, respectively. Since genera of *Cutibacterium* and *Staphylococcus*, *M. globosa*, and *M. restricta* were the most abundant microorganisms in our results, we collected ASVs with these four genera and species and conducted network analysis. As a result, we find out the correlations of *C. acnes* with *Staphylococcus* and *M. globosa* and provide practical microorganism candidates that modulate the microbiomes associated with acne.

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Conflict of Interest

We have no conflicts of interest to report.

Ethical Statement

This project was reviewed by the Institutional Review Board (IRB) of the Korea National Institute for Bioethics Policy (IRB no. IECK(1)-IRB-18K041326), and written consent was provided by all subjects.

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