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Analysis of the Microbiome of the Ear Canal in Normal Individuals and Patients with Chronic Otitis Externa

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Background: Recently, microbiome research has been actively conducted for various skin areas. However, no study has yet compared the microbiome of bacteria and fungi in the ear canal of healthy individuals and patients with chronic otitis externa in Korea.
Objective: This study aimed to investigate the difference in the distribution of fungal and bacterial microbial communities in ear canal samples of healthy individuals and patients with chronic otitis externa.
Methods: In 24 patients with bilateral chronic otitis externa and 24 healthy controls, cotton swabs were used to obtain samples from the bilateral ear canal. To characterize the fungal and bacterial communities, we sequenced and analyzed the 16S rRNA V4–V5 and ITS1 regions using Quantitative Insights into Microbial Ecology 2, respectively.
Results: The alpha diversity analysis for bacteria and fungi confirmed that both richness and evenness decreased in the patient group. The beta diversity analysis for bacteria confirmed that these parameters differed between the control and patient groups. The beta diversity analysis for fungi showed no difference between the groups.
Conclusion: We observed different skin microbiomes in the patients with chronic otitis externa compared with those in the healthy individuals.

Keywords: Bacteria, Ear canal, Fungi, Microbiota, Otitis externa

INTRODUCTION

The human skin hosts numerous commensal and pathogenic microbes, including bacteria, fungi, and viruses[1]. These microbes influence one another, and their composition may change with exposure to the surrounding environment[2].

The human ear canal is approximately 2.5 to 3.0 cm in length and is composed of two parts: distal cartilaginous canal and proximal bony canal[3]. The skin of the distal canal exhibits numerous hair follicles, with approximately 1,000 to 2,000 sebaceous and ceruminous glands (modified apocrine gland)[4]. Histologically, these structures are very similar to those in the outer skin. However, the ear canal is anatomically narrow and can be easily obstructed by ear plugs or debris, which can have properties different from those of the outside, open environment. In addition, a waxy substance, composed of long-chain fatty acids, alcohols, squalene, and cholesterol, is secreted by the ceruminous glands in the ear canal[5]. This waxy substance is known to perform a dual function—(1) reduce the pH of the ear canal to act as an antimicrobial defense mechanism and (2) provide water-repellent qualities to the skin of the ear canal[6-8].

Previous studies have investigated bacteria or selected fungi in disease-free ear canals[9]; however, to our knowledge, no studies have conducted an integrated analysis of bacteria and fungi. In this study, microbial communities of fungi and bacteria from a sample of healthy individuals and patients with chronic otitis externa were analyzed in both ears, and differences in the distribution of the microbiome were examined.

MATERIALS AND METHODS

Participant and sample preparation

Twenty-four patients diagnosed with bilateral chronic otitis externa by an otolaryngologist were recruited. In addition, 24 disease-free individuals, with no history of chronic otitis externa and absence of skin diseases, were recruited as controls.

Individuals in both the patient and control groups diagnosed with any cutaneous infectious disease of the ear canal, including otomycosis through physical examination; those with any systemic immune-associated diseases; those with a history of receiving concomitant systemic or topical (used within 12 weeks of enrollment) treatments that could affect the microbiome results, particularly antimicrobial and antifungal agents, anti-inflammatory drugs, and immunomodulators, including steroids; and those who received treatment using ear drops within 2 weeks before enrollment were excluded.

For all participants, sampling of the ear canal was performed using cotton swabs, under near-sterile conditions, with the researchers wearing gloves and surgical masks. Both ear canals were swabbed approximately 50 times for >30 seconds each. The swabs were stored at −80°C until used for genomic DNA (gDNA) extraction. All participants provided informed consent for inclusion before participation in the study, and the study protocol was approved by our Institutional Review Board (KUH202005011).

Bacterial and fungal gDNA extraction

Bacterial and fungal gDNA was extracted from the cotton swabs using a commercially available kit (PureLink Genomic DNA Mini Kit; Life Technologies, Waltham, MA, USA) and a bead beating method. Initially, the cotton swabs were treated with 400 μl lysozyme digestion buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, lysozyme [20 mg/ml]), and incubated at 37°C in a water bath for 1 hour[10]. Subsequently, 45 μl of proteinase K was added, and then 445 μl of genomic lysis/binding buffer was added to the sample. Using the Bead Beater 16 (Bio Spec Products Inc., Bartlesville, OK, USA), we performed bead beating for 1 minute with two stainless beads (QIAGEN GmbH) at each sample. To remove the heat generated during bead beating, we cooled the samples for 10 minutes on ice and 10 minutes at room temperature, before incubating in a water bath at 55°C for 30 minutes. Subsequently, the bacterial cell lysate from the PureLink Genomic DNA Mini Kit was processed according to the manufacturer's instructions. Once DNA extraction of all samples was completed, the concentration and purity of the gDNA were measured using a spectrophotometer (NanoDrop 2000; Thermo-Fisher Scientific Inc., Waltham, MA, USA).

Target gene amplification for cluster analysis

To examine any significant microbiome differences between the patients with otitis externa and controls, we amplified each target region in the bacterial and fungal genes using polymerase chain reaction (PCR). For bacteria, 518F, 5'–TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCA GCA GCY GCG GTA AN–3' targeting the V4–V5 region and 926R, 5'–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCC GTC AAT TCN TTT RAG T–3' PCR primers were used among the hypervariable regions of the 16S rRNA gene. For fungi, '18S-F, 5'–TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTA AAA GTC GTA ACA AGG TTT C–3' targeting the ITS1 region and 5.8S-R, 5'–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGT TCA AAG AYT CGA TGA TTC AC–3' PCR primers were used.

For gene amplification, gDNA (≥10 ng/μl), each 1 μM PCR primer (i.e., forward and reverse), and KAPA HiFi Hotstart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) were mixed to prepare reaction volume. For PCR, after denaturation (95°C, 3 minutes), denaturation (95°C, 30 seconds), annealing (55°C, 30 seconds), and elongation (72°C, 1 minutes) were repeated (30 cycles for bacteria and 33 cycles for fungi) before the final elongation (72°C for 5 minutes).

Illumina MiSeq sequencing and raw data processing

The amplified 16S rRNA gene and ITS1 region were sequenced using the Illumina MiSeq sequencing platform. In Illumina MiSeq sequencing, sequences are generally read in the forward and reverse directions from both ends of each sequence; from this, two paired-end sequence FASTQ files per sequence are obtained. Herein, a total of 182 paired-end sequence reads from a total of 91 samples were obtained.

Bacterial sequencing using Quantitative Insights into Microbial Ecology 2

Ninety-one bacterial sequencing samples were used for the initial analysis. The plugin for the Quantitative Insights into Microbial Ecology 2 (QIIME2) pipeline was used for the analysis of the skin microbial cluster in the ears of each group. Initially, the Cutadapt plugin was used to remove the 518-forward/926-reverse primer, which was used in the first PCR reaction from the paired-end sequences, and untrimmed sequences were excluded[11]. The sequenc-

es were trimmed using the dada2 denoise-paired plugin, and a common part of the forward and reverse sequences was merged and quality-filtered using Q-score 18. ASVs (amplicon sequence variants) were created, and once representative sequences corresponding to each ASV were selected, a feature table corresponding to the operational taxonomic unit (OTU) table was created.

Taxonomy was assigned to each ASV in the Greengenes database using the feature-classifier classify-sklearn plugin. During sample collection, the human mitochondrial sequence derived from the human skin surface was removed, and the chloroplast sequence, which cannot be derived from humans, was also removed. The sequence of Archaea, which was not the target bacteria/fungi, was also removed.

Alignment was performed using the phylogeny align-to-tree-mafft-fasttree plugin of QIIME2 pipeline. Therefore, we got the samples with a rarefied depth of 900 reads when the sum of the number of reads was calculated after the chloroplast and mitochondria were removed.

Fungal sequencing using Quantitative Insights into Microbial Ecology

UNITE reference OTUs (version 2020.02), which are ITS sequence databases of fungi, were used. As with bacterial sequence analysis, the untrimmed sequences in the fungal sequence analysis were also excluded after removing the 18S-F/5.8S-R primer sequence used in the first PCR reaction using the Cutadapt plugin from the paired-end sequences. After trimming of the sequence using the dada2 denoise-paired plugin, the common part of the forward and reverse sequences was found and merged. Among fungi, the taxonomy was assigned to each ASV in the UniteDB_20.02.02 database using the feature-classifier classify-sklearn plugin. Subsequently, a feature table was created in the same manner as in the bacterial sequencing method. As a result, alignment was also performed as described above for the bacterial communities, and the sequences were rarefied with a depth of 1,083 reads.

Statistical methods

Analysis of similarities (i.e., "ANOSIM") was performed to identify factors that differentiated the microbial communities. The Wilcoxon rank sum test or t-test was performed to determine whether the UniFrac dissimilarity, alpha diversity, and taxonomies of the two groups were significantly different. Alpha diversity was verified using the diversity plugin for the Chao1, Shannon, and Simpson indices. Beta diversity was analyzed using principal coordinate analysis (PCoA).

RESULTS

Twenty-four patients each with chronic otitis externa and with healthy ear canals were recruited. The mean±standard deviation age was 51.5±19.13 years in the patient group and 50.29±14.95 years in the control group (p=0.808). Both groups comprised 9 male and 15 female. There was no significant difference in age and sex between the two groups.

A total of 96 samples were obtained from both ear canals of the patient and control groups. According to the quality control and number of reads, 32 samples from the patient group and 47 from the control group for bacteria and 34 samples from the patient group and 47 from the control group for fungi were included in the final analysis (Fig. 1).

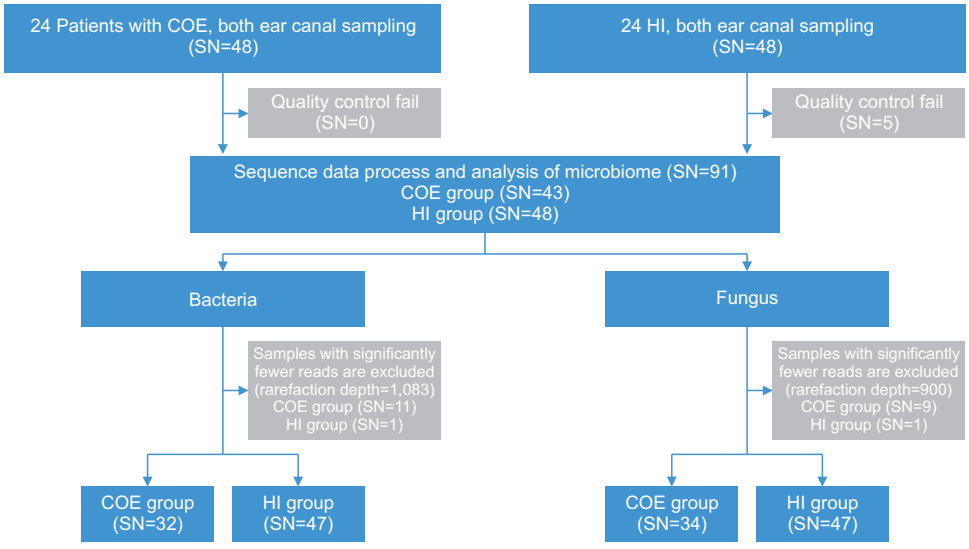


Fig. 1. Flow chart of the study process. COE: chronic otitis externa, HI: healthy individuals, SN: sample numbers.

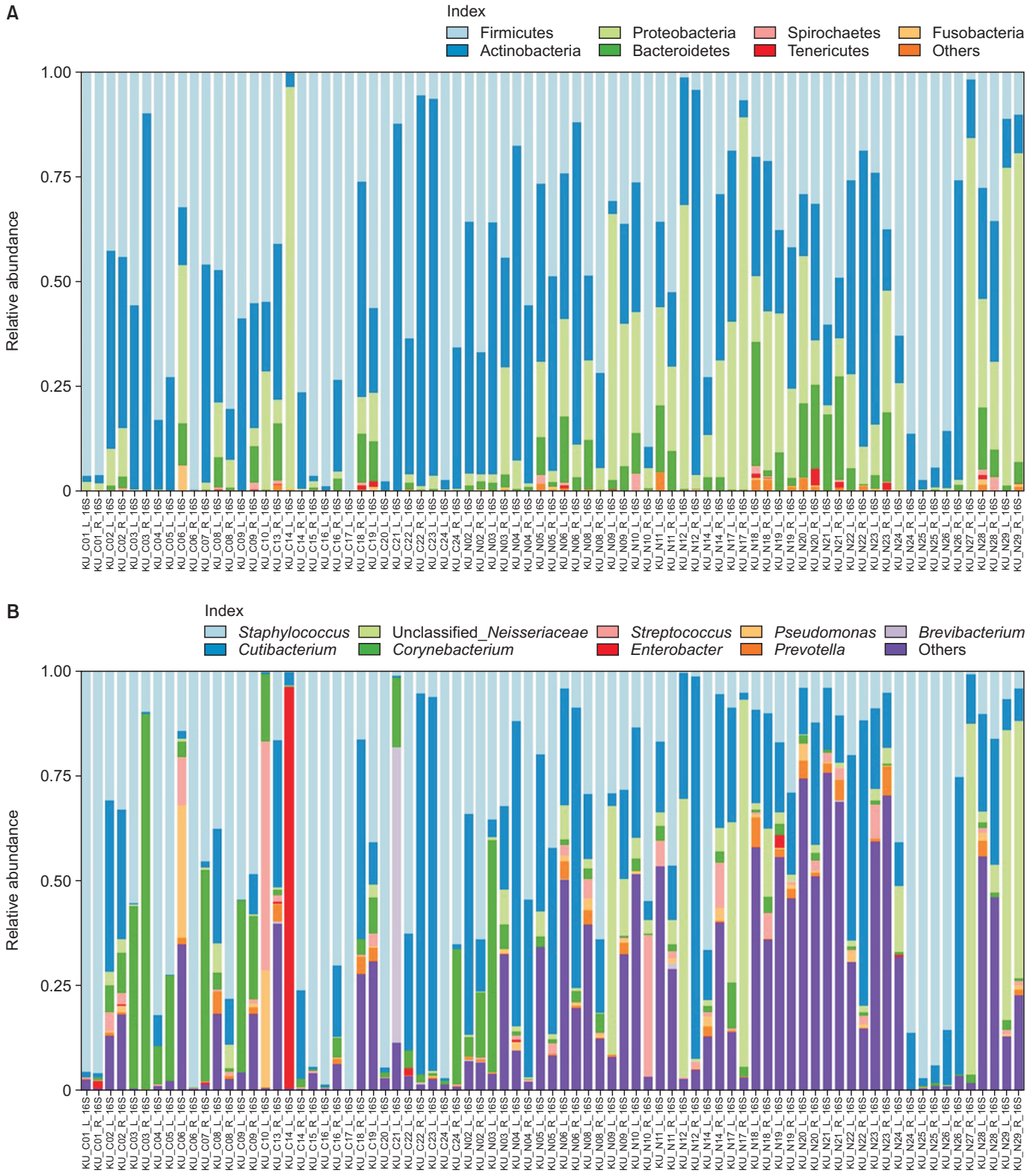
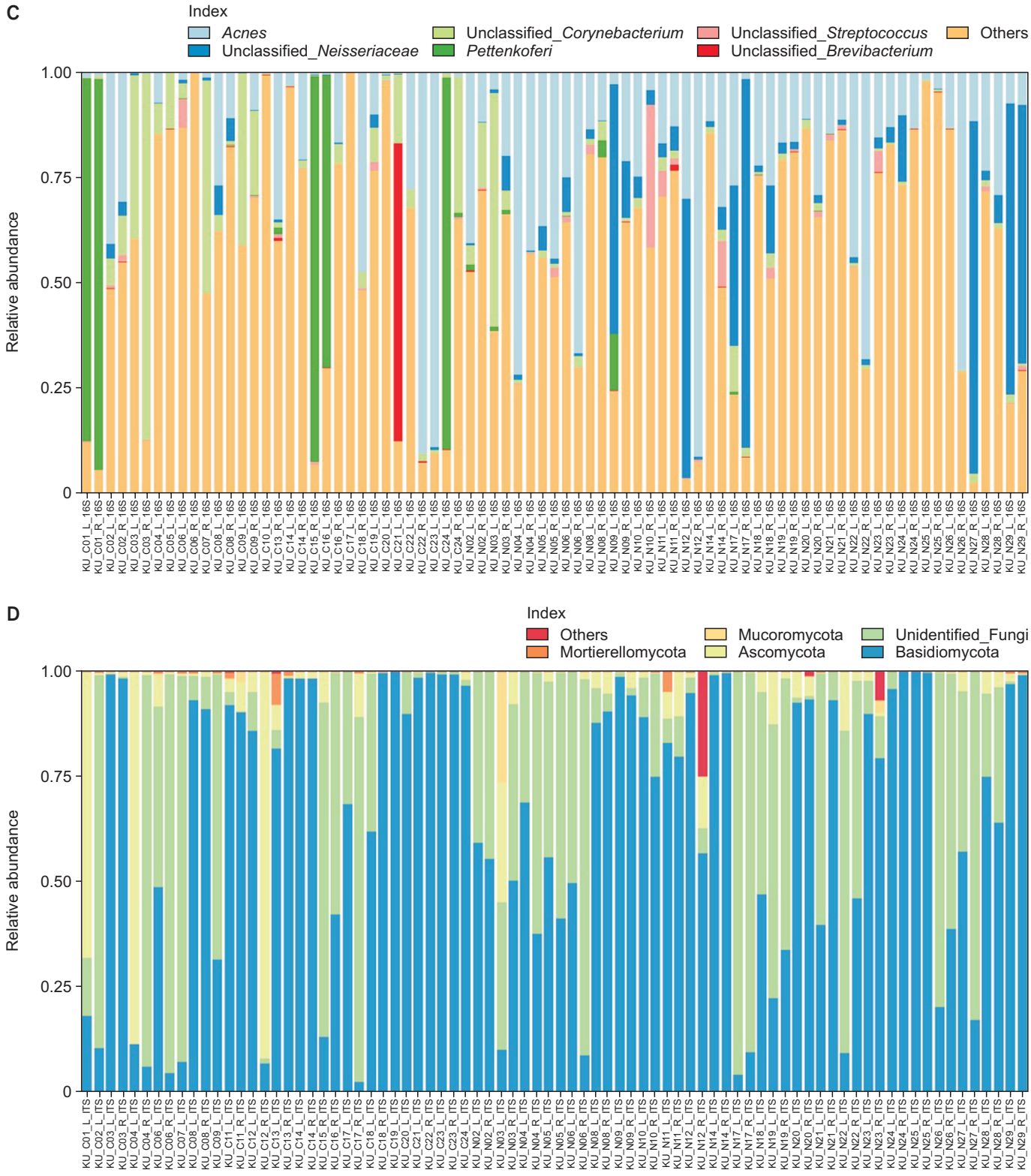


Fig. 2. Taxonomic compositions of the bacterial and fungal communities in the ear canal of the patient and control groups. (A) Relative abundance of the top eight bacteria in the ear canal at the phylum level. (B) Relative abundance of the top 10 bacteria at the genus level. (C) Relative abundance of the top seven bacteria at the species level. (D) Relative abundance of the top six fungi at the phylum level. (E) Relative abundance of the top six fungi at the genus level. (F) Relative abundance of the top six fungi at the species level.



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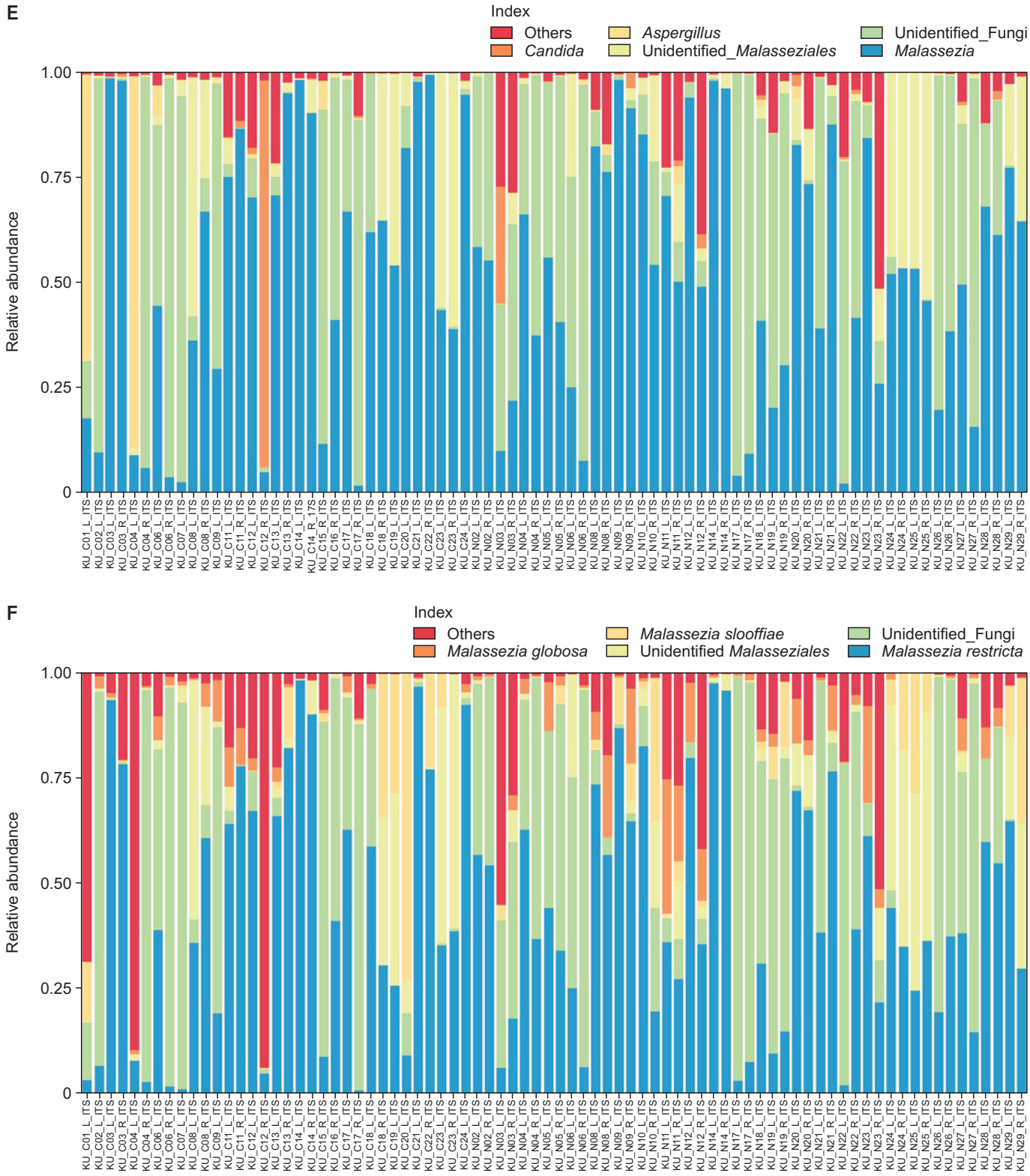

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Taxonomic composition of the skin microorganisms in the patient and control groups

1) Bacterial relative abundance

Bacterial taxonomy analysis was performed for the entire population using the feature-classifier classify-sklearn plugin of QIIME2 and Greengenes database. The analysis revealed a total of 22 phyla, 397 genera, and 534 species.

At the phylum level, Firmicutes accounted for 37.2%, and Actinobacteria accounted for 24.2% of all bacterial clusters. Proteobacteria and Bacteroidetes accounted for 13.2% and 3.5%, respectively (Fig. 2A).

At the genus level, *Staphylococcus* and *Cutibacterium* accounted for most of the bacterial clusters (29.9% and 16.6%, respectively), followed by unclassified *Neisseriaceae* and *Corynebacterium* (6.1% and 5.4%, respectively) (Fig. 2B).

At the species level, other spp. accounted for 45.0%, while *Cutibacterium acnes*, unclassified *Neisseriaceae*, unclassified *Corynebacterium*, and *Staphylococcus pettenkoferi* accounted for 16.2%, 6.1%, 5.0%, and 4.5% of the entire cluster, respectively (Fig. 2C).

2) Fungal relative abundance

Fungal taxonomy analysis was performed for the entire population using the feature-classifier classify-sklearn and the Unite database on the QIIME2 platform. The analysis revealed a total of 5 phyla, 149 genera, and 213 species.

At the phylum level, Basidiomycota accounted for 51.5% of the total fungal clusters. This was followed by unidentified fungi and Ascomycota (22.9% and 4.5%, respectively) (Fig. 2D).

At the genus level, *Malassezia,* unidentified fungi, and *Malasseziales* other than *Malassezia* accounted for 42.3%, 22.9%, and 7.0% of the entire fungal cluster, respectively. *Aspergillus* and *Candida*, known as major pathogens responsible for the onset of otitis externa, were found to be present in small proportions (1.8% and 1.4%, respectively) among all fungal clusters (Fig. 2E).

At the species level, *Malassezia restricta* accounted for the largest proportion (34.7%), followed by unidentified fungi and unidentified *Malasseziales* (22.9% and 6.9%, respectively). *Malassezia slooffiae*, belonging to another genus of *Malassezia*, and *Malassezia globosa* accounted for 4.0% and 2.9%, respectively (Fig. 2F).

The differences in the relative abundances between the patient and control groups are summarized in Table 1.

Table 1. Comparison of the microbiota using permutational multivariate analysis of variance between the patient and control groups

Classification	Patient group (%)	Control group (%)
Bacteria		
Phylum		
Firmicutes	59.10	38.90
Actinobacteria	30.20	31.00
Proteobacteria	7.70	22.80
Bacteroidetes	2.30	5.99
Genus		
Staphylococcus	53.10	27.40
Cutibacterium	15.00	25.00
Unclassified *Neisseriaceae*	0.80	12.60
Corynebacterium	12.40	3.10
Species		
Other species	56.70	57.80
Cutibacterium acnes	15.00	24.00
Unclassified *Neisseriaceae*	0.80	12.60
Unclassified *Corynebacterium*	11.20	3.00
Staphylococcus pettenkoferi	13.40	0.45
Fungi		
Phylum		
Basidiomycota	64.90	64.00
Unidentified fungi	25.20	31.10
Ascomycota	9.20	3.20
Genus		
Malassezia	53.50	52.30
Unidentified fungi	25.20	31.10
Unidentified *Malasseziales*	9.60	8.10
Aspergillus	5.20	0.30
Candida	2.90	0.90
Species		
Malassezia restricta	44.70	42.50
Unidentified fungi	25.20	31.10
Unidentified *Malasseziales*	9.60	8.10
Malassezia slooffiae	5.90	4.40
Malassezia globosa	1.90	4.90

Alpha diversity analysis of the skin microorganisms in the patient and control groups

To confirm alpha diversity, we determined the Chao1, Shannon, and Simpson indices. For bacteria, the Chao1 (p=0.00004) and Shannon indices (p=0.00002), which indicate richness, were found to be significantly low in the patient group; similar-

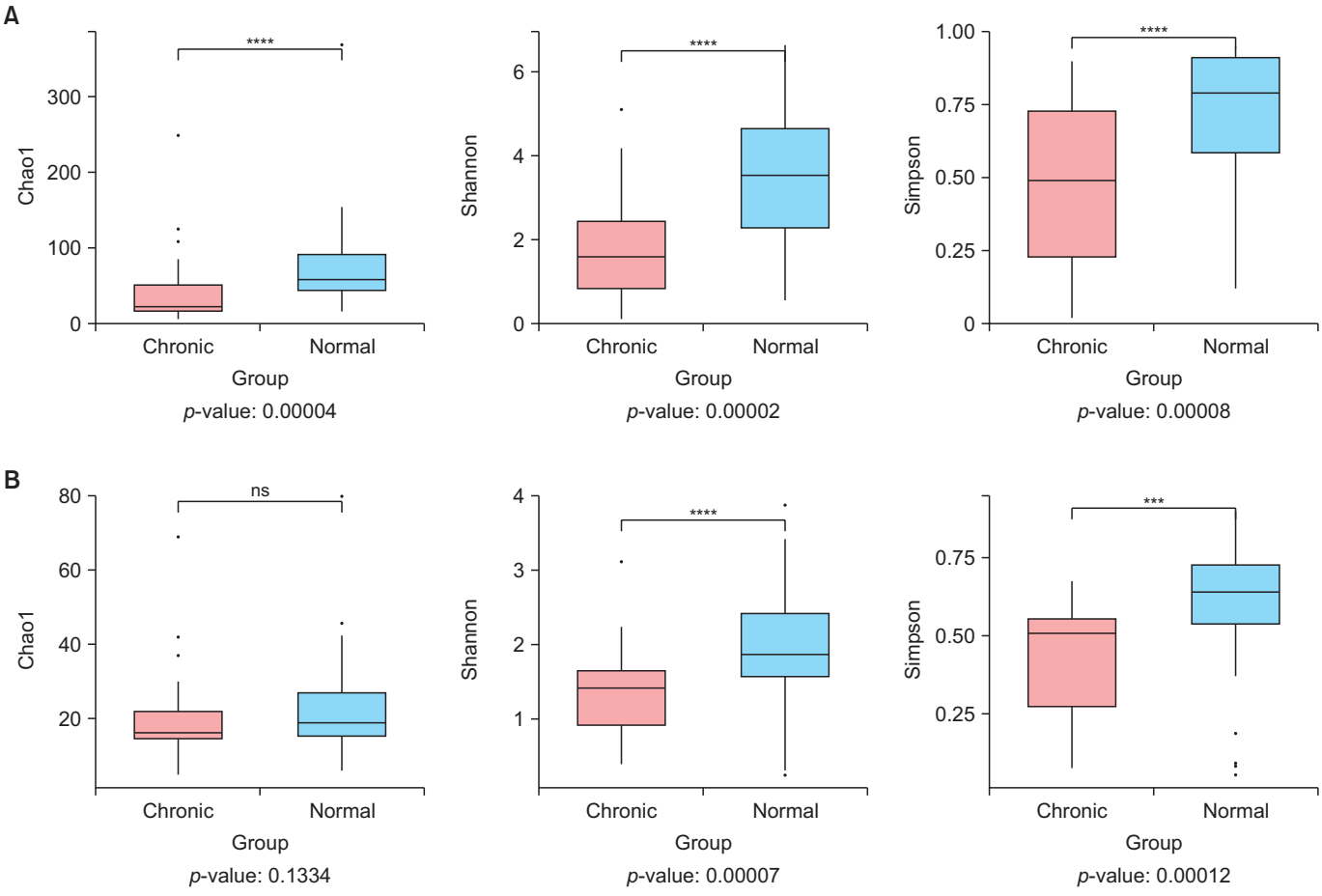

Fig. 3. Comparison of the alpha diversity between the patient and control groups. (A) For bacteria, the Chao1, Shannon, and Simpson indices were significantly low in the patient group. (B) For fungi, there was no significant difference in the Chao1 index, while the Shannon and Simpson indices were significantly lower in the patient group than in the control group. ns: not significant. ***$p<0.001$, ****$p<0.0001$.

ly, the Simpson index (p=0.00008), which indicates evenness, was found to be significantly low in the patient group (Fig. 3A).

For fungi, there was no significant difference in the Chao1 index, while the Shannon index (p=0.00007) was found to be significantly low in the patient group. The Simpson index (p=0.00012) was also found to be significantly low in the patient group (Fig. 3B).

Beta diversity analysis of the skin microbes in the ear canals of the patient and control groups

PCoA was performed to determine the overall characteristics of the patient and control groups. Unweighted UniFrac, a qualitative method that considers the abundance of ASVs, and weighted UniFrac, a quantitative method, were used. For bacteria, significant differences were found in both unweighted (p=0.001) and weighted UniFrac (p=0.004) between the groups (Fig. 4A). For fungi, no significant difference was found between the groups (Fig. 4B).

DISCUSSION

In our study, *Staphylococcus* (27.4%), *Cutibacterium* (25.0%), *Neisseriaceae* (12.6%), and *Corynebacterium* (3.1%) were identified in the ear canals of the healthy individuals. In the previous study by Sjövall et al.[9], the microbial clusters identified in the ear canals of healthy Caucasians included *Staphylococcus auricularis*, *C. acnes*, *Alloiococcus otitis*, and *Turicella otitidis* in decreasing order. Meanwhile, Frank et al.[12] reported that *A. otitis*, *Corynebacterium otitidis*, and *S. auricularis* were the predominantly observed species. The difference in these re-

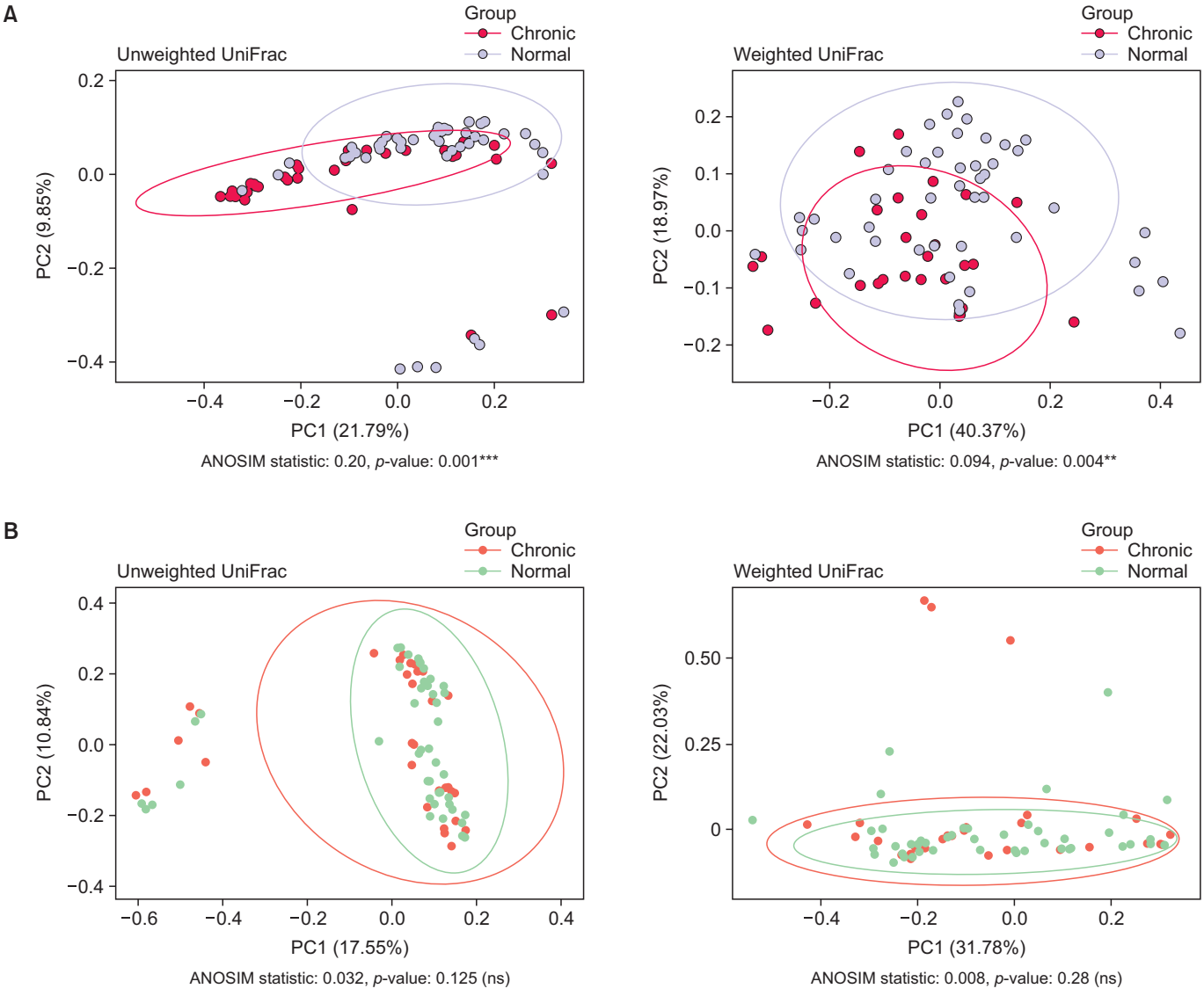

Fig. 4. Principal coordinate analysis of the beta diversity between the patient and control groups. (A) For bacteria, significant differences were found in both unweighted and weighted UniFrac between the groups. (B) For fungi, no significant difference was found in both unweighted and weighted UniFrac. PC: principal coordinate, ns: not significant. $^{**}p<0.01$, $^{***}p<0.001$.

sults can be attributed to the characteristics of the microbiome that change owing to age, race, and environmental factors of the sampled participants[13]. Existing studies that mostly identified *A. otitis* and *T. otitidis* were conducted mostly in children[14], and age-related differences may have affected the results. There may be differences in the internal environment of the ear canal depending on race. More specifically, Europeans and Africans exhibit a thick, moist lipid-rich form of earwax, while Asians exhibit a grayish, dry form of earwax containing significant amounts of protein[8]. The difference in the habitat of these microbes inside the ear may have influenced the results. Owing to differences in research techniques, there may also be a difference in the results; previous studies mostly used 16S rRNA V3–V4 for PCR amplification, in contrast to our study, in which V4–V5 was used.

In our study, *Staphylococcus* and *Cutibacterium* were predominant in both groups at the genus level. However, compared with the control group, the patient group exhibited an increase in *Staphylococcus* and *Corynebacterium*, which are hydrophilic; more specifically, *Staphylococcus* exhibited a two-fold relative share. In contrast, there was a decrease in *Cutibacterium*, which is lipophilic. It is known that the cerumi-

nous gland, a modified form of the sebaceous gland, secretes earwax to block external moisture and regulates internal humidity[8]. It can be inferred that these microbiome changes may have occurred owing to changes in physiological function and gland damage caused by chronic otitis externa.

Unclassified *Neisseriaceae*, which was found in 12.6% of the controls, was found in only 0.8% of the patients; this finding suggests that *Neisseriaceae* spp. could be a significant species contributing to the difference in the microbiome between the patient and control groups.

At the species level, other spp. accounted for 45% of the total clusters; this could be interpreted as mostly unclassified species of *Staphylococcus*, which accounted for the largest proportion in the genus level.

For fungi at the phylum level, Basidiomycota was present in similar proportions in the patient and control groups (64.9% and 64.0%, respectively); this finding indicates that it was the dominant flora present in the ear, regardless of the presence or absence of chronic otitis externa lesions.

At the genus level, *Aspergillus*, a candidate pathogen for inducing chronic otitis externa, was found in 5.2% of the patients and in 0.3% of the controls; *Candida*, another candidate pathogen, was found in 2.9% and 0.9% of the patients and controls, respectively. This finding is consistent with previous reports that *Aspergillus niger* and *Candida albicans* are the most typical pathogens causing otitis externa[15].

In the alpha diversity analysis for bacteria and fungi, it was confirmed that both richness and evenness decreased in the patient group. In the beta diversity analysis for bacteria, it was confirmed that these parameters differed between the control and patient groups. Meanwhile, in the beta diversity analysis for fungi, no difference was found between the groups.

In conclusion, we observed different skin microbiomes in the patients with chronic otitis externa compared with those in the healthy individuals. To the best of our knowledge, this is the first study to investigate the skin microbiome using a culture-independent sequencing method in the ear canal of patients with chronic otitis externa and healthy individuals in Korea.Nevertheless, further research is needed to address the limitations of our study, which include the relatively high number of drop outs during quality control for patients with chronic otitis externa and the non-investigation of specific predisposing factors for ear canal colonization. Despite these limitations, our study could provide a foundation for future research investigating additional diseases associated with the ear canal.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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