ORIGINAL RESEARCH

Cholesteatoma has an altered microbiota with a higher abundance of *Staphylococcus* species

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Abstract

Objective: To compare the microbiota between cholesteatoma and chronic suppurative otitis media (COM) and to identify potential pathogens that explain the relevant phenotypes of cholesteatoma.

Study Design: Prospective cohort study.

Methods: Surgical specimens collected from 20 cholesteatomas and nine COMs were treated to dissolve biofilms and subjected to 16S ribosomal RNA (rRNA) gene sequencing and amplicon sequence variant-level analysis for microbiota profiling and quantitative comparison. Correlations between the relative abundance of potential pathogens and the volume of the primary resected cholesteatomas were examined.

Results: Differences in bacterial composition (beta diversity) were observed between cholesteatomas and COM ($p = .002$), with a higher abundance of *Staphylococcus* in cholesteatomas than in COM ($p = .005$). Common genera in the external auditory canal (EAC) flora, such as *Staphylococcus*, *Corynebacterium*, and *Cutibacterium*, were predominant in both cholesteatoma and COM; *Staphylococcus aureus* and *Pseudomonas aeruginosa* were increased in both diseases compared with the EAC flora. Furthermore, coagulase-negative staphylococci (CoNS) were more abundant in cholesteatomas than in COM ($p = 0.002$). Linear discriminant analysis coupled with effect size measurements (LEfSe) identified four CoNS as potential biomarkers for cholesteatoma. The relative abundance of *S. aureus*, a potential pathogen, was positively correlated with cholesteatoma volume ($r = .60$, $p = .02$).

Conclusion: The microbiota of cholesteatoma and COM originated from EAC flora, but the bacterial composition was largely altered. Our results suggested that *S. aureus* infection is involved in cholesteatoma progression.

Level of Evidence: 3b

1 | INTRODUCTION

The bacterial cells that colonize the skin and mucosa outnumber human cells, and these complex microbial communities have a large impact on human health and disease.[1] The microbiota of the healthy external auditory canal (EAC) is longitudinally stable[2] and well characterized; the dominant commensals are *Staphylococcus auricularis* (abundance:28.7%), *Alloiococcus otitidis* (19.5%), *Turicella otitidis* (18.3%), and *Cutibacterium acnes* (15.4%), while pathogenic bacteria, such as *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, are extremely rare.[3–6] However, when colonizing bacteria migrate through the retraction pocket or a tympanic perforation, they may form biofilms to survive in this abnormal environment, and alter the microbiota.[7,8] Biofilm formation is commonly observed in cholesteatoma keratin debris as well as in the granulation tissues of chronic suppurative otitis media (COM).[9–12] Furthermore, previous studies have suggested that infections may stimulate the aggressiveness of cholesteatoma and increase bone resorption.[13] Although biofilm-forming *S. aureus* and *P. aeruginosa* are recognized as the major pathogens in cholesteatoma and COM based on traditional culture-based studies,[14,15] their microbiota have not been fully characterized. Therefore, a comprehensive analysis of the microbiota is necessary to understand the disease pathogenesis and develop new treatment strategies.

16S ribosomal RNA (rRNA) gene sequencing analysis has been widely used to characterize the diversity and composition of complex microbial communities. Marker gene analysis uses primers that target single or multiple hypervariable regions of the 16S rRNA gene to profile the bacterial composition, generally at genus-level resolution. To the best of our knowledge, four studies have analyzed the bacteria in cholesteatoma and COM using 16S rRNA gene sequencing.[16–19] These studies showed differences in the middle ear microbiota between patients with these conditions and healthy controls, but they did not characterize the relevant microbiota of cholesteatoma and COM. These results could be attributed to inappropriate DNA extraction protocols and bioinformatics pipelines used for data analysis. Biofilm microorganisms are embedded in a stable matrix of extracellular polymeric substances (EPS). Notably, none of the studies included procedures to dissolve EPS and remove polysaccharides and other matrix components, which can bind to nucleic acids during the DNA extraction process and hamper subsequent downstream analysis.[20] In addition, all four studies used operational taxonomic unit (OTU)-level workflows (QIIME,[21] USEARCH, and Ion Reporter) for bioinformatic pipelines. Several studies suggest that these pipelines produce more spurious OTUs and show lower specificity compared with the more recently developed amplicon sequence variant (ASV)-level workflow.[22–24]

Here, we present microbiota analysis of cholesteatoma and COM using a validated biofilm dissolving procedure and high-resolution ASV-level 16S rRNA gene amplicon sequencing. We also attempted to correlate the relative abundance of potential pathogens with cholesteatoma volume.

2 | METHODS

2.1 | Patient recruitment and sampling

The study was approved by the Institutional Review Board of Tokyo Medical and Dental University for Clinical Research (M2017-021), and written informed consent was obtained from the study participants or their parents if the patient was a child. Between 2017 and 2019, 31 patients with cholesteatoma and 18 patients with COM with tympanic membrane perforation were recruited and scheduled to undergo tympanoplasty, with or without cholesteatoma resection. During the surgical procedure, cholesteatoma matrices containing keratin debris or diseased tympanic membranes from patients with COM were carefully resected to prevent contamination and immediately placed in a sterilized tube on ice and then stored at −80°C until processing. In cases of ear discharge, sterile rayon-tipped swab (Copan, 116C) samples were taken from the EAC before surgery and sent to a hospital laboratory for bacterial culture analysis. Swab samples were inoculated on the following culture media: chocolate II agar (Becton Dickinson), trypticase soy agar with 5% sheep blood (Becton Dickinson), modified Drigalski agar (Eiken Chemical), potato dextrose agar with chloramphenicol (Kohjin Bio), and CHROMagar Candida (Kanto Chemical) under aerobic conditions, and Brucella HK agar (Kyokuto) under anaerobic conditions. Significant bacterial species were identified using MALDI Biotyper (Bruker), and antibiotic sensitivity testing was performed using Microscan WalkAway (Beckman Coulter).

2.2 | DNA extraction and 16s rRNA gene sequencing

We used a method combining chemical and mechanical lysis by bead beating. The tissue specimens were transferred to ZR BashingBead Lysis Tubes (2.0 mm Beads; Zymo Research) containing lysis buffer (BF1) from the PowerBiofilm DNA Isolation Kit (MoBio Laboratories) and mechanically homogenized using a Precellys 24 homogenizer (Bertin Technologies) at 6400 rpm for 30–60 s. Total genomic DNA (derived from both humans and bacteria) was extracted according to the manufacturer's instructions. To target a broad range of taxa, six hypervariable regions (V2, V3, V4, V6, V7, V8, and V9) were simultaneously amplified by polymerase chain reaction (PCR) using the Ion 16S Metagenomics Kit (Thermo Fisher), which was validated using mock samples.[25] PCR products (209–295 bases in length)[26] were

observed in each reaction using electrophoresis. Some samples, especially from COM cases, required an increased amount of template DNA and a higher number of PCR cycles (up to 30), according to the manufacturer's instructions. Negative controls containing extraction reagents were used to test for contamination. The amplicons were purified using an Agencourt AMPure XP (Beckman Coulter) and then quantified and analyzed for quality/integrity using a 2100 Bioanalyzer (Agilent Technologies). Libraries were prepared using the Ion Ampli-Seq Fragment Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Sequencing was performed using an Ion S5 system with an Ion 530 chip (Thermo Fisher Scientific). Sequencing data after primer trimming are available at BioProject under accession number PRJNA782304.

2.3 | Data processing

Sequence quality trimming, ASV inference, and taxonomic classification were performed using DADA2 v1.18.0 in RStudio v1.3.1093. Fastq files were generated using Torrent Suite software v5.10.0 (Thermo Fisher Scientific). Using the *filterAndTrim(maxLen = 300, trimLeft = 20, minLen = 180)* script, reads shorter than 200 bases were removed, and the first 300 bases were used in the analysis (for quality profiles, see Figures S1 and S2). The first 20 bases were removed for primer trimming because the primer sequences are not published.[25] For the trimmed data, error model learning, ASV inference, and chimera removal were performed with the default settings.[23] Taxonomy was assigned to the resulting ASVs using Silva v138.1 SSU Ref NR 99 as the rRNA reference database,[27] which is validated for middle ear microbiota analysis.[28] Sequence alignment with the National Center for Biotechnology Information (NCBI) database using BLASTn identified 1132 ASVs of *Homo sapiens* origin, which were removed.[29] ASVs from chloroplasts (35 ASVs) and mitochondria (152 ASVs) were also excluded.[30]

2.4 | Microbiota analysis

Overall changes in the microbiota of cholesteatoma and COM were assessed by alpha and beta diversity using the R packages phyloseq v1.34.0, and vegan v2.5–7. All samples were rarefied to 170,742 sequences (90% of the minimum sample depth). Alpha diversity quantifies the microbial diversity within individual samples. Higher numbers of unique taxa (richness) and more even abundance distributions (evenness) yield larger alpha diversity values. The observed ASVs and Chao1, which reflect the richness, and Shannon and inverse Simpson indices, which consider both richness and evenness, were measured. Statistical differences between groups were tested by the Wilcoxon rank-sum test with Bonferroni–Holm correction. The beta diversity determines the differences in the overall taxonomic composition of each sample pair. The difference in species abundance between samples is measured as a distance, ranging between 0 and 1, with higher numbers representing lower similarity. Pairwise comparisons for a set of samples are presented in a distance matrix. Beta diversity was calculated using Bray–Curtis dissimilarity index, visualized with a Nonmetric Multidimensional Scaling plot, and compared with permutational multivariate analysis of variance. The abundant genera in cholesteatoma and COM were identified using the *relAbundChart* script.[6] To identify the bacteria that explain the greatest differences in taxonomic composition between cholesteatoma and COM, linear discriminant analysis (LDA) coupled with effect size measurements (LEfSe) was performed with default settings[31] using normalized relative abundance values, which were calculated at the species level as described previously.[32] An LDA logarithmic score for each bacterium means a magnitude of statistically consistent difference between cholesteatoma and COM.

2.5 | Cholesteatoma volume measurement

Preoperative temporal bone computed tomography (CT) scan with a 0.5 mm section thickness was performed for all patients, and magnetic resonance imaging (MRI) was performed in cases of cholesteatoma suggestive of mastoid cavity involvement on CT or extracranial complications (Stage II and higher of the EAONO/JOS staging system[33]). MRI was performed on a Titan 3 T unit using a 32-channel head coil (Toshiba). The following protocol was used: 3 mm axial fast spin-echo T2-weighted sequences (time to recovery (TR) = 4800 ms, time to echo (TE) = 90 ms, flip angle = 90°, field of view (FOV) = 200 mm, and number of acquisitions = 2) and 3 mm transverse diffusion (b = 1000 s/mm^2)-weighted images (DWI) with single-shot SEEPI sequence (TR = 4600 ms, TE = 90 ms, flip angle = 90°, FOV = 200 mm, and number of acquisitions = 2). CT and MRI DICOM image stacks were imported into 3D Slicer v.5.0.3[34] and overlaid on each other using the landmark registration protocol (Figure S3). A cholesteatoma was diagnosed if the lesion showed high signal intensity in comparison to surrounding regions of low signal intensity on the b1000 DWI.[35,36] Subsequently, the cholesteatoma was manually segmented with a modification based on surgical findings, and the volume was calculated using ITK-SNAP v.3.4.0.[37] Pearson's correlation coefficient was calculated using R.

3 | RESULTS

3.1 | Patient demographics and bacterial isolate culture

PCR products were not obtained from 7 (23%) of 31 cholesteatoma samples and 5 (28%) of 18 COM samples. The success of DNA amplification was not consistent with the size of the surgical specimens. The remaining samples were sequenced, and four cholesteatomas and four COMs were excluded because of the extremely low number of sequences (mean ± SD:1554 ± 2842 reads per sample) and low-quality scores (<30) on the DADA2 inspection. Finally, 20 cholesteatomas (65% of the initial samples) and nine COMs (50% of the initial samples) were subjected to further analysis. The study participants

	Cholesteatoma	COM	*p*-value
No. of patients	20	9	
Female	7 (35)	5 (56)	.42
Age	45 [7–92]	72 [42–85]	.11
Ear discharge	14 (70)	9 (100)	.14
Cultured taxa[a]			
Staphylococcus aureus	2 (20)	4 (67)	.12
CoNS	8 (80)	1 (17)	.04
Corynebacterium	3 (30)	2 (33)	1.00
Pseudomonas aeruginosa	0	2 (33)	.13
Stenotrophomonas maltophilia	1 (10)	0	1.00
Achromobacter xylosoxidans	0	1 (17)	.38
Candida	1 (10)	2 (33)	.52

TABLE 1 Patient demographics and pathogens cultured from ear discharge

Note: Data are presented as *n* (%) or median [range].
Abbreviations: COM, chronic suppurative otitis media; CoNS, coagulase-negative staphylococci.
[a]Bacterial culture of ear discharge was obtained from 10 patients with cholesteatoma and 6 with suppurative chronic otitis media.

showed no significant differences in demographic characteristics between the two groups (Table 1). Cultures were obtained from 10 patients with cholesteatoma and six patients with COM. *Staphylococcus* species were the most common isolates, and coagulase-negative staphylococci (CoNS) were more common in cholesteatomas than in COMs (80% and 17%, respectively, *p* = .04). *P. aeruginosa* was isolated only from COMs. *The S. aureus* and *P. aeruginosa* isolates were susceptible to common antibiotics, including penicillin and cephem.

3.2 | Microbiota characteristics

The final dataset included 24,863,539 sequence reads (857,363 ± 439,386 reads per sample) and 9654 ASVs. Of these, 9144 ASVs (95%) were assigned at the genus level, and 4917 ASVs (51%) were assigned at the species level. First, we assessed the overall patterns of microbiota variation by calculating alpha and beta diversities. Alpha diversity in cholesteatoma was lower in terms of both richness (observed ASVs, *p* = .72; Chao1, *p* = .80) and evenness (Shannon, *p* = .94; Simpson, *p* = .91), but the differences were not statistically significant (Figure S4). However, beta diversity was significantly different (Figure 1; *p* = .002), suggesting dissimilarities in the microbiota between cholesteatoma and COM.

We then examined the relative abundances of specific taxa in each patient. The bacterial compositions of cholesteatoma and COM were similar at the phylum level (Figure 2A). The microbiota consisted of four phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Firmicutes and Actinobacteria were predominant in cholesteatoma, while Proteobacteria was more common in COM. The larger proportions of Actinobacteria and Firmicutes were similar to the skin microbiota.[38] Notably, the relative abundance of the genus *Staphylococcus* (Firmicutes) was obviously higher in cholesteatoma, which may have influenced the difference in beta diversity.
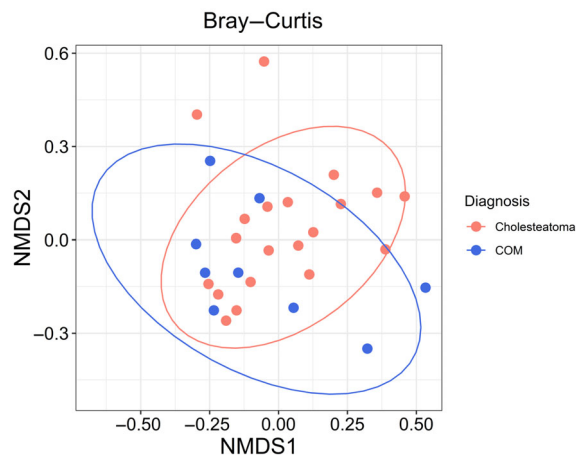

FIGURE 1 Nonmetric multidimensional scaling (NMDS) of cholesteatoma and COM samples. Each dot indicates an individual sample, and dots closer to one another indicate higher similarity. Ellipses represent the 90% confidence intervals. The groups are significantly different (permutational multivariate analysis of variance; *p* = .002). COM, chronic suppurative otitis media

To determine the similarities and differences in bacterial composition between the groups, we compared the mean relative abundances of the most common genera (Table 2). In cholesteatoma, the most abundant taxa were *Staphylococcus* (58.8%), followed by Actinobacteria, including *Corynebacterium* (9.5%), *Brevibacterium* (7.3%, mostly *Brevibacterium otitidis*), and *Cutibacterium* (5.7%, mostly *C. acnes*). In COM, the most abundant taxa were *Corynebacterium* (31.9%, mostly *Corynebacterium striatum*), *Staphylococcus* (17.3%), and *Pseudomonas* (11.2%, mostly *P. aeruginosa*). *Staphylococcus* was more abundant in the cholesteatoma group (*p* = .005), whereas *Corynebacterium* was more abundant in the COM group (*p* = .03). *P. aeruginosa* and *B. otitidis* were more abundant in both diseases relative to the EAC flora

FIGURE 2 Bacterial taxonomic classifications in cholesteatoma and chronic suppurative otitis media (COM). (A) Relative abundances of the major four phyla and three genera of Firmicutes (*Staphylococcus*, *Streptococcus*, and *Clostridia*). (B) Relative abundances of staphylococci at the species level. The order of study subjects is the same as in (A)
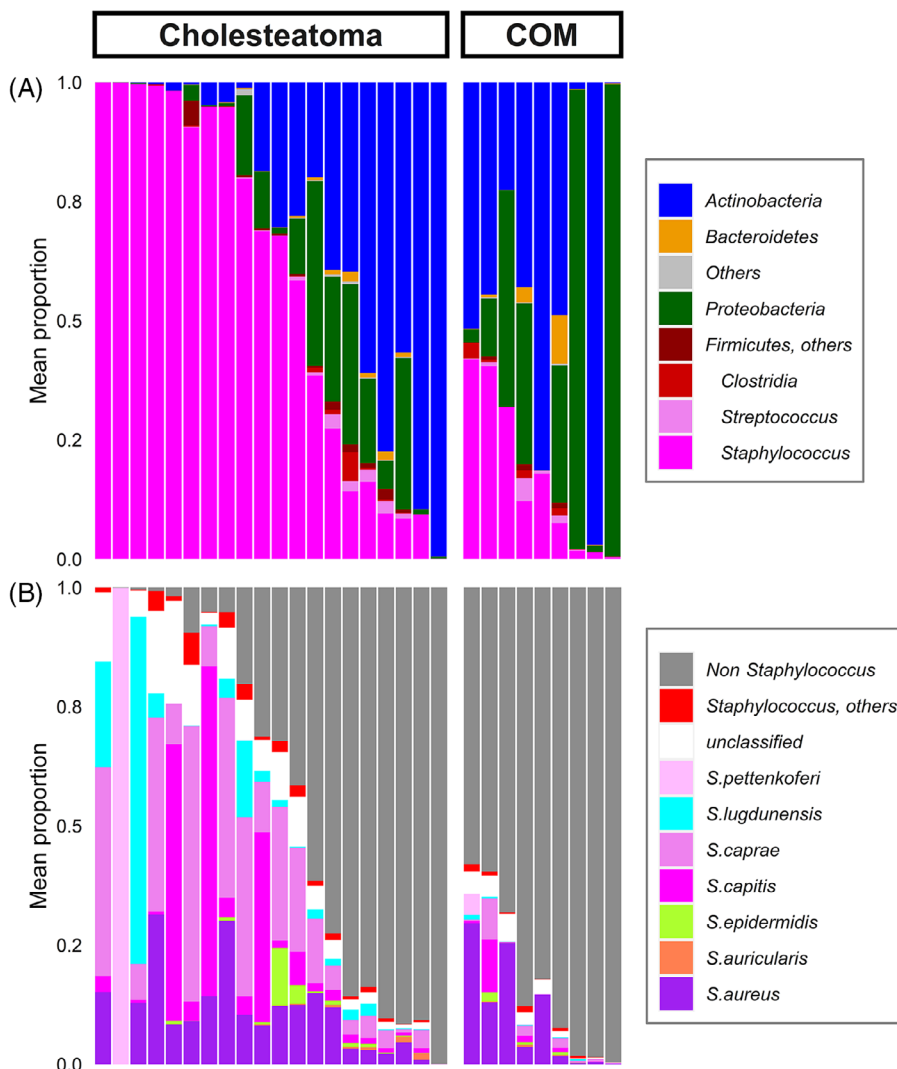

TABLE 2 Mean relative abundance of the top nine genera

	Cholesteatoma (%)	COM (%)	*p*-value
Staphylococcus	58.8	17.3	.005
Corynebacterium	9.5	31.9	.03
Cutibacterium	5.7	6.2	.89
Brevibacterium	7.3	1.8	.46
Pseudomonas	0.6	11.2	.14
Neisseria	0.2	10.0	.13
Acinetobacter	2.5	2.8	.84
Stenotrophomonas	2.0	1.6	.81
Cupriavidus	1.7	1.0	.62
Other taxa	11.0	14.5	.64
Unclassified taxa	0.7	1.7	.28

Abbreviation: COM, chronic suppurative otitis media.

(0.05% and 0.04%, respectively).[6] In contrast, common bacteria in acute otitis media, such as *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *A. otitidis*, and *T. otitidis*,[28,39] were extremely rare (<0.05%) in samples from both diseases.

Considering the high abundance of *Staphylococcus* spp. in cholesteatomas and its pathogenic importance in biofilm-related infections,[13,40,41] we further characterized *Staphylococcus* at the species level (Figure 2B). ASV-level analysis identified ∼90% of the staphylococci. *S. aureus* was present in both cholesteatoma and COM at higher abundances (10% and 9.9%, respectively) than in the EAC flora (<2%).[3] Moreover, the mean relative abundance of CoNS was significantly higher in cholesteatoma than in COM (41% and 5.0%, respectively; *p* = .002). Using LefSe, four CoNS species, *S. caprae*, *S. capitis*, *S. pettenkoferi*, and *S. lugdunensis*, were identified as potential biomarkers for cholesteatoma, with high LDA scores (>4.0; Figure 3). In contrast, *S. auricularis* was extremely rare in both cholesteatoma and COM (Figure 2B) compared with the EAC flora (28.7%).[6]

3.3 | Cholesteatoma volume and bacterial abundance

The study included 16 patients who underwent primary cholesteatoma resection. Using these samples, we examined the relationship between the relative abundance of the potential pathogens identified

above and cholesteatoma volume. The relative abundance of *S. aureus* was positively correlated with cholesteatoma volume (Figure 4A; $r = .60$, $p = .008$). However, no relationship was found between cholesteatoma volume and either CoNS or *P. aeruginosa* (Figure 4B).

4 | DISCUSSION

Although the biofilm-forming bacteria *S. aureus* and *P. aeruginosa* are recognized as the major pathogens in cholesteatoma and COM, the

microbiotas have not been fully characterized. Using more sensitive methods, we characterized the microbiota of cholesteatoma and COM at the species level. In both diseases, the predominant EAC commensals were dramatically decreased, whereas *S. aureus* and *P. aeruginosa* were substantially increased. *Staphylococcus* was more abundant in cholesteatomas, and the abundance of *S. aureus* was correlated with cholesteatoma volume.

4.1 | Comprehensive revision of the ear microbiota analysis methodology

In this study, we reassessed the validity of previously reported 16S rRNA gene sequencing for bacterial identification in cholesteatoma. We revised some processes to avoid possible biases that may influence the results. First, we used tissue samples instead of swabs from the middle ear membrane[16,17] because the cholesteatoma sac is not continuous with the middle ear. Subsequently, we performed chemical lysis combined with mechanical lysis using bead-beating for DNA extraction. Mechanical lysis with bead beating increases the isolation of gram-positive bacteria in Actinobacteria and Firmicutes.[42,43] Furthermore, the PowerBiofilm method that we used includes chemical lysis conditions that remove biofilm matrix components, which has advantages over the previous methods in DNA yield, purity, and integrity.[20] We then analyzed the sequence data using the ASV-level pipeline, DADA2, with the Silva as a reference database, validated in the ear microbiome analysis.[28] The ASV method distinguishes sequence variants differing by a single nucleotide and has superior sensitivity, specificity, and resolution compared with OTU-level analysis.[22–24]
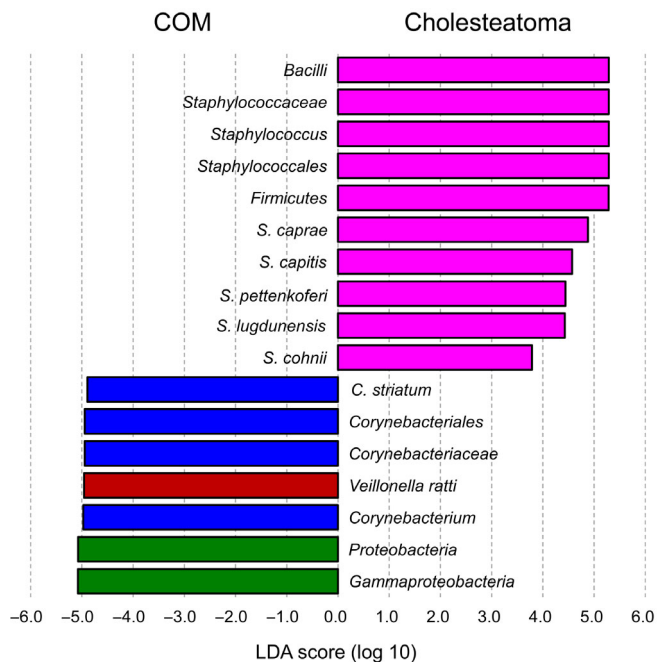

FIGURE 3 Linear discriminant analysis (LDA) coupled with effect size measurements analysis of cholesteatoma compared with COM. The LEfSe analysis identified 17 taxa that are significantly more abundant in one group. The width of each bar represents the effect size of the taxon in the particular group. Red, blue, and green colors indicate the phyla Firmicutes, Actinobacteria, and Proteobacteria, respectively. Statistical significance was set to $p < .05$

4.2 | Altered microbiotas in cholesteatoma and COM

No viable bacterial cells were detected in the healthy middle ear,[44] hence the bacteria associated with otitis media are considered to originate from the EAC skin or nasopharyngeal flora. The most common
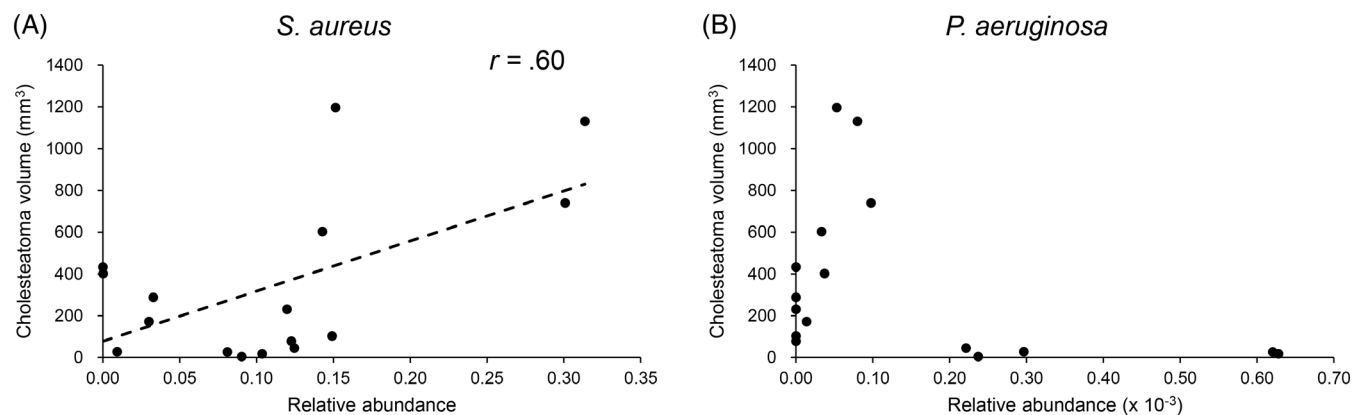

FIGURE 4 Correlations between the relative abundances of pathogenic bacteria and the volume of cholesteatoma

genera identified in our study, *Staphylococcus*, *Corynebacterium*, *Cutibacterium*, *Brevibacterium*, and *Acinetobacter*, are primary colonizers of the skin,[38] including the EAC.[3,4,6] In contrast, the common bacteria in the nasopharynx, such as *Moraxella*, *Streptococcus*, and *Fusobacterium*,[45] were extremely rare in our samples, suggesting that the EAC serves as the major bacterial reservoir for cholesteatoma and COM. However, we observed obvious differences in the bacterial compositions between the flora in a healthy EAC and the microbiotas of both cholesteatoma and COM. The microbiotas of cholesteatoma and COM showed increased colonization by *S. aureus* and *P. aeruginosa*, and dramatic decreases in *S. auricularis*, *A. otitidis*, and *T. otitidis*, which predominantly colonize the EAC and can be easily eradicated by antibiotics.[3] The recovered *S. aureus* and *P. aeruginosa* strains were also susceptible to common antibiotics, which is inconsistent with the limited disappearance of *S. auricularis*, *A. otitidis*, and *T. otitidis* in patients with these diseases. A similar alteration in the EAC microbiota has been reported in acute otitis externa.[46] Moreover, a reciprocal relationship between *S. aureus* and *A. otitidis* has been observed in the middle ear fluid of patients with otitis media,[39] suggesting that *A. otitidis* may compete with pathogenic *S. aureus* for colonization of the EAC. Similarly, the decrease in *T. otitidis* may reflect the increase in closely related *Corynebacterium* species for the same niche, such as *C. striatum* in COM.[28]

Our results show that abundant CoNS is characteristic of cholesteatoma. *Staphylococcus lugdunensis* is distinct from other CoNS due to its high pathogenicity, which is similar to that of *S. aureus*,[40] suggesting its pathogenic potential, at least in some cases of cholesteatoma. Another explanation for the high abundance of CoNS is colonization resistance against *S. aureus* infection. Colonization resistance is the overgrowth of commensal bacteria that contribute to host defense against pathogens in the same niche.[47] *Staphylococcus caprae*, *S. capitis*, and *S. lugdunensis* have been reported to secrete effector molecules that inhibit *S. aureus* colonization and reduce its virulence.[48,49] The distinct CoNS colonization in cholesteatoma may reflect the presence of *S. aureus* strains with higher virulence capacity, although a strain-level analysis was not conducted in this study. *Corynebacterium striatum* was identified as a potential biomarker for COM. This species is component of the skin microbiota, can cause severe nosocomial infections and develop multidrug resistance.[50,51]

4.3 | *Staphylococcus aureus* infection in cholesteatoma and clinical implications

Several studies have suggested that receptor activator of nuclear factor κB ligand (RANKL) plays an important role in inflammatory bone resorption in cholesteatoma.[13,52] Although *P. aeruginosa* lipopolysaccharide can induce osteoclastogenesis and bone resorption,[53] its role in cholesteatoma is controversial because the biofilm factors were not correlated with cholesteatoma growth in an in vivo model,[54] and RANKL expression by matrix-keratinocytes is not affected in the absence of lipopolysaccharide stimulation.[52] In contrast, we found that *S. aureus* was the common taxa in cholesteatoma lesions, and the

relative abundance was correlated with cholesteatoma volume, suggesting the major pathogenic role in cholesteatoma. High prevalence of *S. aureus* in cholesteatomas is consistent with previous culture-based studies.[14,15] Once the infection is established in tissues, *S. aureus* forms biofilms and expresses various toxins and immune evasion factors that promote tissue destruction and chronic inflammation. Protein A, a vilurence factor of *S. aureus*, can stimulate RANKL-mediated osteoclastogenesis and bone resorption through the Immunoglobulin G-binding capacity.[55–57] Furthermore, recent shotgun metagenomic sequencing of cholesteatoma specimens showed that *Aspergillus* is the most common microbe and a potential pathogen for severe bone resorption.[58] Fungus infection was not considered in our study using 16S rRNA sequencing. Long-read sequencing of 16S rRNA gene and internal transcribed regions would provide more accurate information on microbial community, including fungi, with a higher taxonomic resolution.

Because cholesteatomas are easy to access through the EAC, biofilm destruction using bacteriophages and phage-derived products can be a therapeutic strategy against *S. aureus* infection. Bacteriophages infect bacterial cells with high specificity and secret endolysins that enzymatically degrade bacterial peptidoglycan, resulting in cell lysis and biofilm clearance without the undesirable killing of commensals or host tissue degradation, thus avoiding inner ear damage.[41] A single application of a bacteriophage cocktail containing DRA88 and bacteriophage K efficiently reduced *S. aureus* biofilm biomass by up to 6%.[59]

5 | LIMITATIONS

Our study has several limitations. First, considering the interindividual diversity in the EAC microbiota,[6] contralateral healthy EAC tissues would be more appropriate controls. Instead, we compared the microbiota of cholesteatoma with that of COM to examine alterations in the EAC flora and identify potential pathogens in cholesteatoma. Second, in the absence of standardized methods for bacterial DNA extraction from human tissues, we used the Power-Biofilm DNA Isolation Kit with increased bead beating to facilitate the recovery of bacterial DNA from biofilm samples. However, amplification efficiency of ~60% indicated that the protocol needs to be optimized to increase the bacterial-to-host DNA ratio. Our protocol did not include selective lysis of human cells followed by human DNA removal before bead-beating, which is necessary for bacterial DNA enrichment.[43] Adding *N*-acetylcysteine to dissolve biofilms can also improve the taxonomic analysis of the microbiome in cholesteatoma samples.[60] Furthermore, gram-negative bacteria, including *P. aeruginosa*, are possibly lost due to the chaotropic buffer used in the PowerBiofilm DNA Isolation Kit,[42] which can underestimate their significance in cholesteatoma. Third, variations in rRNA gene copy numbers can influence the observed abundance of taxa in a bacterial community. While the variation in rRNA gene copy number increases with phylogenetic distance, quantification of different staphylococci is considered compatible because the variation within species is relatively rare.[61]

6 | CONCLUSION

We present a microbiota analysis of cholesteatoma and COM using improved methods, including bacterial DNA extraction from biofilms and an ASV-level pipeline, which enabled us to identify taxa at species-level resolution using 16S rRNA gene sequencing. While the microbiotas appeared to originate from the EAC flora, the bacterial compositions in cholesteatoma and COM were significantly different from the EAC flora. Microbiome analysis with further optimized methods for both DNA extraction and sequencing is necessary to understand the pathogenesis and develop therapeutic strategies for cholesteatomas.

CONFLICT OF INTERESTS
The authors declare that they have no conflicts of interest.

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REFERENCES

1. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, Knight R. Current understanding of the human microbiome. *Nat Med*. 2018; 24(4):392-400.
2. Grice EA, Kong HH, Conlan S, et al. Topographical and temporal diversity of the human skin microbiome. *Science*. 2009;324(5931):1190-1192.
3. Stroman DW, Roland PS, Dohar J, Burt W. Microbiology of normal external auditory canal. *Laryngoscope*. 2001;111(11 Pt 1):2054-2059.
4. Frank DN, Spiegelman GB, Davis W, Wagner E, Lyons E, Pace NR. Culture-independent molecular analysis of microbial constituents of the healthy human outer ear. *J Clin Microbiol*. 2003;41(1):295-303.
5. Lappan R, Jamieson SE, Peacock CS. Reviewing the pathogenic potential of the otitis-associated bacteria *Alloiococcus otitidis* and *Turicella otitidis*. *Front Cell Infect Microbiol*. 2020;10:51.
6. Sjövall A, Aho VTE, Hyyrynen T, et al. Microbiome of the healthy external auditory canal. *Otol Neurotol*. 2021;42(5):e609-e614.
7. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol*. 2016;14(9):563-575.
8. Kong HH. Skin microbiome: genomics-based insights into the diversity and role of skin microbes. *Trends Mol Med*. 2011;17(6): 320-328.
9. Chole RA, Faddis BT. Evidence for microbial biofilms in cholesteatomas. *Arch Otolaryngol Head Neck Surg*. 2002;128(10):1129-1133.
10. Lampikoski H, Aarnisalo AA, Jero J, Kinnari TJ. Mastoid biofilm in chronic otitis media. *Otol Neurotol*. 2012;33(5):785-788.
11. Gu X, Keyoumu Y, Long L, Zhang H. Detection of bacterial biofilms in different types of chronic otitis media. *Eur Arch Otorhinolaryngol*. 2014;271(11):2877-2883.
12. Galli J, Calò L, Giuliani M, et al. Biofilm's role in chronic cholesteatomatous otitis media: a pilot study. *Otolaryngol Head Neck Surg*. 2016; 154(5):914-916.
13. Kuo CL. Etiopathogenesis of acquired cholesteatoma: prominent theories and recent advances in biomolecular research. *Laryngoscope*. 2015;125(1):234-240.
14. Mittal R, Lisi CV, Gerring R, et al. Current concepts in the pathogenesis and treatment of chronic suppurative otitis media. *J Med Microbiol*. 2015;64(10):1103-1116.
15. Xu F, Kong W, Peng J, Gu H, Zheng H. Analysis of main pathogenic bacteria and drug sensitivity in patients with chronic suppurative otitis media and middle ear cholesteatoma in China. *Biotechnol Lett*. 2020;42(8):1559-1166.
16. Neeff M, Biswas K, Hoggard M, Taylor MW, Douglas R. Molecular microbiological profile of chronic suppurative otitis media. *J Clin Microbiol*. 2016;54(10):2538-2546.
17. Minami SB, Mutai H, Suzuki T, et al. Microbiomes of the normal middle ear and ears with chronic otitis media. *Laryngoscope*. 2017; 127(10):E371-E377.
18. Kalcioglu MT, Guldemir D, Unaldi O, Egilmez OK, Celebi B, Durmaz R. Metagenomics analysis of bacterial population of tympanosclerotic plaques and cholesteatomas. *Otolaryngol Head Neck Surg*. 2018; 159(4):724-732.
19. Weiss JP, Antonelli PJ, Dirain CO. Microbiome analysis of cholesteatoma by gene sequencing. *Otol Neurotol*. 2019;40(9):1186-1193.
20. Corcoll N, Österlund T, Sinclair L, et al. Comparison of four DNA extraction methods for comprehensive assessment of 16S rRNA bacterial diversity in marine biofilms using high-throughput sequencing. *FEMS Microbiol Lett*. 2017;364(14):fnx139.
21. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37(8):852-857.
22. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J*. 2017;11(12):2639-2643.
23. Xue Z, Kable ME, Marco ML. Impact of DNA sequencing and analysis methods on 16S rRNA gene bacterial community analysis of dairy products. *mSphere*. 2018;3(5):e00410-18.
24. Prodan A, Tremaroli V, Brolin H, Zwinderman AH, Nieuwdorp M, Levin E. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. *PLoS One*. 2020;15(1):e0227434.
25. Barb JJ, Oler AJ, Kim HS, et al. Development of an analysis pipeline characterizing multiple hypervariable regions of 16S rRNA using mock samples. *PLoS One*. 2016;11(2):e0148047.
26. Thermo Fisher Scientific Inc. 16S rRNA Sequencing. https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/Ion-16S-Metagenomics-Kit-Software-Application-Note.pdf; 2014.
27. McLaren MR, Callahan BJ. Silva 138.1 prokaryotic SSU taxonomic training data formatted for DADA2 [Dataset]. https://zenodo.org/record/4587955 Published March 7, 2021. Accessed November 16, 2021.
28. Lappan R, Imbrogno K, Sikazwe C, et al. A microbiome case-control study of recurrent acute otitis media identified potentially protective bacterial genera. *BMC Microbiol*. 2018;18(1):13.
29. US National Library of Medicine. Nucleotide BLAST. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome Published July 13, 2021. Accessed November 16, 2021.
30. Knight R, Vrbanac A, Taylor BC, et al. Best practices for analysing microbiomes. *Nat Rev Microbiol*. 2018;16(7):410-422.
31. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12(6):R60.
32. Kostic AD, Gevers D, Pedamallu CS, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res*. 2012;22(2):292-298.
33. Yung M, Tono T, Olszewska E, et al. EAONO/JOS joint consensus statements on the definitions, classification and staging of middle ear cholesteatoma. *J Int Adv Otol*. 2017;13(1):1-8.

34. Fedorov A, Beichel R, Kalpathy-Cramer J, et al. 3D slicer as an image computing platform for the quantitative imaging network. *Magn Reson Imaging*. 2012;30(9):1323-1341.

35. Venail F, Bonafe A, Poirrier V, Mondain M, Uziel A. Comparison of echo-planar diffusion-weighted imaging and delayed postcontrast T1-weighted MR imaging for the detection of residual cholesteatoma. *AJNR Am J Neuroradiol*. 2008;29(7):1363-1368.

36. Plouin-Gaudon I, Bossard D, Ayari-Khalfallah S, Froehlich P. Fusion of MRIs and CT scans for surgical treatment of cholesteatoma of the middle ear in children. *Arch Otolaryngol Head Neck Surg*. 2010;136(9):878-883.

37. Yushkevich PA, Pashchinskiy A, Oguz I, et al. User-guided segmentation of multi-modality medical imaging datasets with ITK-SNAP. *Neuroinformatics*. 2019;17(1):83-102.

38. Grice EA, Segre JA. The skin microbiome. *Nat Rev Microbiol*. 2011;9(4):244-253.

39. Sillanpää S, Oikarinen S, Sipilä M, et al. *Moraxella catarrhalis* might be more common than expected in acute otitis media in young Finnish children. *J Clin Microbiol*. 2016;54(9):2373-2379.

40. Becker K, Heilmann C, Peters G. Coagulase-negative staphylococci. *Clin Microbiol Rev*. 2014;27(4):870-926.

41. Schilcher K, Horswill AR. Staphylococcal biofilm development: structure, regulation, and treatment strategies. *Microbiol Mol Biol Rev*. 2020;84(3):e00026-19.

42. Biesbroek G, Sanders EA, Roeselers G, et al. Deep sequencing analyses of low density microbial communities: working at the boundary of accurate microbiota detection. *PLoS One*. 2012;7(3):e32942.

43. Bruggeling CE, Garza DR, Achouiti S, Mes W, Dutilh BE, Boleij A. Optimized bacterial DNA isolation method for microbiome analysis of human tissues. *Microbiol Open*. 2021;10(3):e1191.

44. Jervis-Bardy J, Leong LEX, Papanicolas LE, et al. Examining the evidence for an adult healthy middle ear microbiome. *mSphere*. 2019;4(5):e00456-19.

45. De Boeck I, Wittouck S, Wuyts S, et al. Comparing the healthy nose and nasopharynx microbiota reveals continuity as well as niche-specificity. *Front Microbiol*. 2017;8:2372.

46. Roland PS, Stroman DW. Microbiology of acute otitis externa. *Laryngoscope*. 2002;112(7 Pt 1):1166-1177.

47. Lawley TD, Walker AW. Intestinal colonization resistance. *Immunology*. 2013;138(1):1-11.

48. Paharik AE, Parlet CP, Chung N, et al. Coagulase-negative staphylococcal strain prevents *Staphylococcus aureus* colonization and skin infection by blocking quorum sensing. *Cell Host Microbe*. 2017;22(6):746-756.e5.

49. Chin D, Goncheva MI, Flannagan RS, et al. Coagulase-negative staphylococci release a purine analog that inhibits *Staphylococcus aureus* virulence. *Nat Commun*. 2021;12(1):1887.

50. Kang SJ, Choi SM, Choi JA, et al. Factors affecting the clinical relevance of *Corynebacterium striatum* isolated from blood cultures. *PLoS One*. 2018;13(6):e0199454.

51. Silva-Santana G, Silva CMF, Olivella JGB, et al. Worldwide survey of *Corynebacterium striatum* increasingly associated with human invasive infections, nosocomial outbreak, and antimicrobial multidrug-resistance, 1976–2020. *Arch Microbiol*. 2021;203(5):1863-1880.

52. Iwamoto Y, Nishikawa K, Imai R, et al. Intercellular communication between keratinocytes and fibroblasts induces local osteoclast differentiation: a mechanism underlying cholesteatoma-induced bone destruction. *Mol Cell Biol*. 2016;36(11):1610-1620.

53. Zhuang L, Jung JY, Wang EW, et al. *Pseudomonas aeruginosa* lipopolysaccharide induces osteoclastogenesis through a toll-like receptor 4 mediated pathway in vitro and in vivo. *Laryngoscope*. 2007;117(5):841-847.

54. Chole RA, Gagnon PM, Vogel JP. Inactivation of specific *Pseudomonas aeruginosa* biofilm factors does not alter virulence in infected cholesteatomas. *Otol Neurotol*. 2014;35(9):1585-1591.

55. Kassem A, Lindholm C, Lerner UH. Toll-like receptor 2 stimulation of osteoblasts mediates *Staphylococcus aureus* induced bone resorption and osteoclastogenesis through enhanced RANKL. *PLoS One*. 2016;11(6):e0156708.

56. Ren LR, Wang ZH, Wang H, He XQ, Song MG, Xu YQ. *Staphylococcus aureus* induces osteoclastogenesis via the NF-κB signaling pathway. *Med Sci Monit*. 2017;23:4579-4590.

57. Kamohara A, Hirata H, Xu X, et al. IgG immune complexes with *Staphylococcus aureus* protein a enhance osteoclast differentiation and bone resorption by stimulating fc receptors and TLR2. *Int Immunol*. 2020;32(2):89-104.

58. Jiang H, Wu C, Xu J, et al. Bacterial and fungal infections promote the bone erosion progression in acquired cholesteatoma revealed by metagenomic next-generation sequencing. *Front Microbiol*. 2021;12:761111.

59. Alves DR, Gaudion A, Bean JE, et al. Combined use of bacteriophage K and a novel bacteriophage to reduce *Staphylococcus aureus* biofilm formation. *Appl Environ Microbiol*. 2014;80(21):6694-6703.

60. Dinicola S, De Grazia S, Carlomagno G, Pintucci JP. N-acetylcysteine as powerful molecule to destroy bacterial biofilms. A systematic review. *Eur Rev Med Pharmacol Sci*. 2014;18(19):2942-2948.

61. Louca S, Doebeli M, Parfrey LW. Correcting for 16S rRNA gene copy numbers in microbiome surveys remains an unsolved problem. *Microbiome*. 2018;6(1):41.

SUPPORTING INFORMATION

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