Microbiome of the Healthy External Auditory Canal

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Objective: To investigate the microbiota of the healthy external auditory canal (EAC) culture-independently and to evaluate the usefulness of the swabbing method in collecting EAC microbiota samples.

Study Design: Cohort study.

Patients: Fifty healthy asymptomatic working-age volunteers.

Intervention: Samples were harvested with DNA-free swabs from the volunteers’ EACs.

Main Outcome Measures: Amplicon sequencing of the 16S rRNA gene was used to characterize the microbial communities in the samples.

Results: The swabbing method is feasible for EAC microbiota sample collection. The analyzed 41 samples came from 27 female and 14 male subjects; 4 samples were excluded due to recent antimicrobial treatment and 5 because of low sequence count or suspected contaminant microbes. The four most frequent amplicon sequence variants in the microbiota data were Staphylococcus auricularis, Propionibacterium acnes, Alloiococcus otitidis, and Turicella otitidis. Typically, the dominant amplicon sequence variant in a sample was one of the most frequent bacteria, but there were also subjects where the dominant species was not among the most frequent ones. The genus Alloiococcus was least common in females who reported cleaning their ears. Subjects with a high relative abundance of Alloiococcus typically had a low abundance of Staphylococcus, which may be a sign of the two being competing members of the microbial community.

Conclusions: The most common bacteria in the microbiome of the healthy EAC were Staphylococcus auricularis, Propionibacterium acnes, Alloiococcus otitidis, and Turicella otitidis. The EAC microbiota seems more diverse and individualized than previously thought. Also, ear cleaning habits seem to alter the EAC microbiome.

Key Words: 16S rRNA gene sequencing—Bacteria—External auditory canal—Microbiome—Microbiota.

Otol Neurotol 42:e609–e614, 2021.

The human body harbors numerous microorganisms, including bacteria, viruses, fungi, and archaea, which form microbiotas that together with their environment form microbiomes (1). In a normal, healthy situation this host–microbe interaction is mutualistic, beneficial to both microbe and host (2). The human body contains many habitats for microorganisms differing considerably in humidity, temperature, exposure to sunlight, acidity, and quantity of sebum. These factors lead to significant intrapersonal divergence in the microbiomes in different parts of the human body (3). The human microbiome is unique to every individual. It begins to develop during birth and stabilizes in the first years of life with exposure to the microorganisms of the surrounding environment (4). The compositions of microbiomes are affected by many factors, such as the host genome, age, sex, family members’ microbiotas, nutrition, and hormones. Usually the healthy adult microbiome remains stable (5–7).

The human microbiome has been described as the second genome because of its colossal effects on health and disease (8,9). Dysbiosis, a disturbance of the microbiome, caused e.g. by antimicrobials, damage to the epithelium or altered immunological defense mechanisms, may lead to disease of the host (10). It is hypothesized that the loss of environmental biodiversity may affect human microbiota and increase the prevalence of asthma, allergies, and other inflammatory diseases.
Gut microbiome alterations have been reported in association with diseases such as Parkinson’s disease, primary sclerosing cholangitis, ulcerative colitis, and colorectal cancer (13–16). A bidirectional connection linking the gut and skin, referred to as the skin-gut axis, has also been demonstrated (17). Local skin dysbiosis, loss of microbial diversity, and changes in the skin-gut axis have been linked to several skin diseases, such as atopic eczema, acne vulgaris, and psoriasis (18–21).

The microorganisms of the body have traditionally been examined by cultivation-based methods. Since unknown proportions of bacteria can be cultivated, this approach only reveals a minority of all bacteria. Novel, culture-independent methods such as targeted sequencing of the 16S ribosomal RNA gene have revolutionized microbiome research (22). These approaches enable detection of microbes directly from environmental samples. The 16S ribosomal RNA gene, present in all bacteria and archaea, contains species- and even strain-specific hypervariable regions that can be used for taxonomic classification, and this combination of universality and specificity has made it the most prevalent genetic marker for surveying microbial communities (23,24). In microbiota analyses, we can explore the organismal diversity by using alpha and beta diversity indices. Alpha diversity illustrates sample richness alone (how many different taxa) or combined with evenness (how evenly the taxa are distributed). Beta diversity demonstrates the dissimilarity of two or more given samples (25). With the current bioinformatic tools, amplicon sequence variants (ASVs), corresponding to a species or even a strain present in the sequenced microbial sample, can be identified (26).

The average length of the human external auditory canal (EAC) is 2.5 cm. The outer third consists of cartilage and is covered with thick and mobile skin. This cartilaginous part of the EAC contains sebaceous and ceruminous apocrine glands and hair follicles. The inner two-thirds form a bony part where the skin is thin, immobile, and contains no glands or hair follicles; this skin is continuous with the epithelium of the tympanic membrane. The EAC has a unique self-cleaning mechanism, where the sloughed epithelium slowly migrates outward to the membranous part and mixes with glandular secretions to form cerumen (27). Cerumen has two distinct types: 1) wet-type, common in Europeans and Africans, brown and sticky, rich in lipids and 2) dry-type, prevalent in East Asians, grayish and rich in protein (28). The role of cerumen is disputed, from only expelling the slough epithelium—to various functions such as moistening, lubrication, protection, and acting as an antimicrobial by maintaining the acidic environment of the EAC (27–29).

Traditionally, staphylococci and coryneforms were assumed to be the predominant colonizers of the EAC (30). In a newer culture-independent study, staphylococci were the most abundant taxon, followed by coryneforms and streptococci (31). The few culture-independent studies suggest Staphylococcus otitis, Corynebacterium ostitidis, and Staphylococcus auricularis as the three dominating commensal microbes in the healthy EAC (32,33). In recent years, a growing number of next-generation sequencing-based microbiome studies have deepened the understanding of health and disease of many human organs. However, the microbiome of the EAC remains scarcely examined. To better understand the pathologies behind different ear diseases, deepened knowledge regarding microbiota of the normal EAC is needed. In the future, this knowledge may enable development of new treatments, including modulation of the EAC microbiome with probiotics or transplantation of healthy microbiome. The aim of this study is to investigate the microbiome of the healthy EAC.

**METHODS**

The study design was approved by the ethical committee of Helsinki University Hospital. Fifty healthy Caucasian volunteers (15 [30%] male), aged 22 to 63 years (mean: 37; median: 34), were recruited in 2018 (20 of them in March, 30 in September). The volunteers were staff from the Helsinki University Hospital Department of Otorhinolaryngology (37 subjects) and medical students (13 subjects) from the University of Helsinki. All volunteers signed an informed consent form and filled an inquiry of their health status including data of their medication, skin diseases, allergies, previous ear problems, tympanic membrane perforations, hearing aids and other ear apparatus in use, ear cleaning habits, swimming and water protection habits, ear drop use, current ear symptoms, current upper respiratory symptoms and current fever. Exclusion criteria were: any significant ear disease, current ear symptoms or existing dermatological disease, as well as antimicrobial or corticosteroid treatment (peroral, intravenous or topical ear steroid) in the past month. Four volunteers had received antimicrobial treatment within a month of sampling and were excluded from further analyses, thus 46 subjects were included at the start of the analyses. A sterile DNA-free sample stick (4N6FLOQSwabs, Thermo-Fisher Scientific) was gently rotated in the cartilaginous part of each volunteer’s EACs. The tip of the stick was stored in a sterile DNA-free Eppendorf tube. Both ears were sampled separately. The tubes were frozen to −72 Celsius until thawing for DNA extraction. After the sample donation, otomicroscopy of both ears was performed to evaluate the auricle, EAC, and tympanic membrane and to note any anatomical aberration, irritation of skin, moisture or dryness in the EAC, abundance or lack of cerumen, secretion, stenosis, and tympanomeatal angle blunting. Other visible skin areas were inspected for signs of eczema.

DNA extraction, PCR amplification, and sequencing (Illumina MiSeq platform) of V3 to V4 variable regions of the 16S rRNA gene were performed in the DNA Sequencing and Genomics Laboratory of the Institute of Biotechnology, University of Helsinki, using a previously published protocol (34) and primers (13). In all laboratory steps, the samples were handled in two batches, with the samples collected in March and those collected in September analyzed separately. Sequence quality trimming, inferring ASVs, and taxonomic classification were done with DADA2, v. 1.12.1 (35). Potential contamination during sampling or laboratory handling was estimated with decontam, v. 1.8.0 (36), and based on this, 56 ASVs were removed as suspected contaminants, along with 61 ASVs as chloroplasts or cyanobacteria. After this trimming, five samples were excluded due to a low number of sequence reads or a high proportion of suspected contaminants. The final data contained 8,878,042 sequence reads (mean ± SD: 108,269 ± 30,875 reads per sample) and 3,650 ASVs. A more detailed description of the bioinformatic analysis is provided in the supplement (eMaterials, http://links.lww.com/MAO/B176).
and the raw sequences have been deposited in the European Nucleotide Archive with the accession number PRJEB34644.

Statistical analyses were performed in R v. 3.6.0 (37), mainly using the packages phyloseq v. 1.28.0 (38) and vegan v. 2.5–6 (39). Alpha diversity measures (observed richness, Shannon, and inverse Simpson) were contrasted using the Wilcoxon signed-rank test (paired samples) or the Wilcoxon rank-sum test (unpaired comparisons). Beta diversity was explored using Bray-Curtis dissimilarity, visualized with Non-Metric Multi-dimensional Scaling, and compared with PERMANOVA. Clustering by community type was performed following the enterotyping tutorial (in brief, the JSD distance metric and the Partitioning Around Medoids algorithm were used for clustering, and the optimal number of clusters chosen based on the Calinski-Harabasz (CH) Index and the Silhouette coefficient) (40). Differential abundance was tested with DESeq2 (41). Full analysis details are available as supporting information (eMaterials in the supplement, http://links.lww.com/MAO/B176).

RESULTS

The final data included 27 (66%) female and 14 (34%) male subjects (Table 1). Out of them, 18 (44%) volunteers, 15 (83%) of them females, mentioned a habit of cleaning their ears with cotton swabs (Q-tips or similar). Three (7%) reported using earplugs while sleeping. Sixteen (39%) reported having had a cold within the past month, including ongoing cases.

Since the two separate sample collection and laboratory handling batches could lead to noticeable differences, the potential microbial community differences between these batches were tested before any comparisons of clinical details. There were no statistically significant differences in alpha or beta diversity between the two batches, although plots suggested some minor differences (eFigure 1 in the supplement, http://links.lww.com/MAO/B177).

Another notable grouping variable in the data was left or right ear. The results did not support a difference when contrasting all right and left ears in the data: there were no statistically significant differences in alpha or beta diversity between the two groups, although plots suggested some minor differences (eFigure 2 in the supplement, http://links.lww.com/MAO/B178). There were also no differences in alpha or beta diversity when contrasting samples depending on whether they represented the side of the subject’s dominant hand or not (p > 0.66 for all alpha diversity indices; p = 0.99 for beta diversity). To simplify the statistical comparisons, all following analyses were focused on right ear samples.

The four most frequent ASVs in the microbiota data were classified as Staphylococcus auricularis, Propionibacterium acnes, Alloiococcus otitis, and Turicella otitidis (eFigure 3 in the supplement, http://links.lww.com/MAO/B179). There were clear differences between subjects regarding the dominant ASV or genus; typically, it was one of the largest four, but there were also some subjects where none of the four were particularly common. To further explore these apparent differences in overall community type, we tested clustering the samples similarly to the suggested enterotypes used for gut microbiota. Tests for optimal number of clusters (CH index and mean silhouette) suggested four, five, or six clusters; we focused on five clusters, which had a slightly higher CH index than four or six. The resulting clusters seemed quite distinct (Fig. 1). Cluster 1 was dominated by Staphylococcus, cluster 2 by Alloiococcus, cluster 3 by Propionibacterium, cluster 4 by Turicella, and cluster 5 by both Staphylococcus and Turicella (in contrast to cluster 1, in which subjects typically had a low relative abundance (percentage of a specific microbe in a sample) of Turicella).

All 41 samples contained sequences of Staphylococcus, Alloiococcus, and Propionibacterium, and 40 (98%) samples also contained Turicella (eTable 1 in the supplement, http://links.lww.com/MAO/B180). Staphylococcus was overall the most abundant genus, with a mean relative abundance of 39.46% (SD: 33.15%). Regarding the less typical taxa, ASVs classified as Pseudomonas were found in 17 (41%) of the samples; however, their overall abundance was very low, with a mean of 0.05% (SD: 0.12%).

Regarding the otomicroscopy of the ears, the findings were minor with no statistically significant discoveries. Contrasting diversity and variables of interest, focusing only on right ear samples, there were no differences in alpha diversity. However, there was a difference in beta diversity between subjects who clean their ears with swabs and those who do not (p = 0.02), and sex was close to significant (p = 0.07) (Table 2). Since the ear

| Variable              | Females (n = 27) | Males (n = 14) |
|-----------------------|------------------|----------------|
| Age (mean ± SD)       | 36.96 ± 10.23    | 38.21 ± 12.45  |
| Allergy               | No: 11, yes: 16  | No: 9, yes: 5  |
| Ear cleaning swab     | No: 12, yes: 15  | No: 11, yes: 3 |
| Earplugs              | No: 24, yes: 3   | No: 14, yes: 0 |
| Handedness            | Left: 3, right: 24 | Left: 2, right: 11, not available: 1 |
| Past ear infections   | No: 23, yes: 4   | No: 10, yes: 3, not available: 1 |
| Recent common cold    | No: 15, yes: 12  | No: 9, yes: 4, not available: 1 |
| Sampling season       | Autumn: 8, spring: 19 | Autumn: 6, spring: 8 |
cleaning and sex variables were unevenly distributed, with only three males who reported cleaning their ears, we also tested a three-group variable excluding the ear-cleaning males, and comparing 1) females who clean their ears (n = 15), 2) females who do not clean their ears (n = 12), and 3) males who do not clean their ears (n = 11) (Fig. 2 and Table 2). This three-group variable was significant on its own (p = 0.03) and when corrected for sampling batch (p = 0.02). DESeq2 was used to test what specific bacterial taxa differ between these three groups in a model corrected for season/batch. The most notable difference detected was for ASV3 (Alloiococcus otitis), which was the most abundant in males and the least abundant in the females who reported cleaning their ears with swabs (eFigure 4 in the supplement, http://links.lww.com/MAO/B181; full results tables of the DESeq2 comparisons in the eMaterials file).

**DISCUSSION**

The aim of this analysis was to investigate the microbiome of the healthy EAC. To our knowledge, this study
is the largest culture-independent analysis of the healthy EAC microbiome to date. The most common bacterial species inhabiting the healthy EAC were Staphylococcus auricularis, Propionibacterium acnes, Alloiococcus otitidis, and Turicella otitidis. Earlier studies also suggested Staphylococcus, Alloiococcus, and Turicella as colonizers of the healthy EAC (30–32). In addition, we propose Propionibacterium as the fourth genus of the healthy EAC microbiome. Earlier topographical studies support these findings showing that sebaceous skin sites are predominated by propionibacteria and staphylococci, while corynebacteria prefer moist sites (3). Considering the less aerated environment of the EAC, with sebaceous and ceruminous apocrine glands in its lateral third, the findings of this study are logical. Other qualities of the EAC such as wet/dry cerumen, pH, humidity, ceruminal biomarkers, and their effect on the microbiome deserve further investigation.

Frank et al. (32) did not find S. auricularis and A. otitidis in the same samples and suggested that these microbes may compete with each other in the EAC. We found Staphylococcus and Alloiococcus in most of the samples, but in a community type clustering analysis, the samples were often dominated by either Staphylococcus, Alloiococcus, Propionibacterium, or Turicella. Samples with a high relative abundance of Staphylococcus typically had a low abundance of Alloiococcus and vice versa, which is in line with the hypothesis of the two as competing members of the microbial community. Compared with the present study, Frank et al. in 2003 used older tools with a lower coverage of sequences, and therefore might not have detected less abundant taxa.

Harvesting the samples and freezing them in −72°C until thawing for DNA extraction functioned expectedly, as most samples yielded sufficiently data and had low proportion of suspected contaminants. There is also likely to be some seasonal variation in the microbial community, and earlier studies have shown such variation in acute otitis externa (42). Unfortunately, the seasonal variation of the healthy EAC microbiota could not be confirmed in this study. Although plots suggested some minor differences between the samples from spring and autumn, the difference between the two sets of samples might be a technical batch effect. In future studies, all samples need to be treated as one batch.

Our group of 41 subjects was dominated by females (66%). There was a difference in overall microbial community composition (beta diversity) between females and males. Additionally, Alloiococcus was most common in males, and least common in females who cleaned the ears. This indicates that ear canal manipulation, and possibly hormonal levels, alter the EAC microbiome. Differences in the microbial composition of left and right EACs, even when considering handedness, were statistically insignificant. Age did not modulate the microbiota either, in our working-age subjects.

Limitations of this study include: 1) relatively small sample size, 2) majority of volunteers were health care personnel, 3) seasonal variation could not be confirmed due to the possible batch effect.

**CONCLUSION**

In line with previous studies, our data demonstrates that the most common genera of the healthy EAC include Staphylococcus, Alloiococcus, and Turicella. Our data further identified Propionibacterium as the fourth genus. In addition, many other bacteria colonize the healthy EAC, and the normal flora is unique in every individual. Interestingly, manipulation of the EAC and sex seem to alter the EAC microbiome. To develop novel EAC microbiome modulating treatments, further studies with larger sample sizes, and different patient groups, are needed.

**Acknowledgments:** The authors thank all the volunteers participating in this study, and the personnel of the DNA sequencing and genomics laboratory for performing the NGS assays.

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