Identification of a dynamic middle ear microbiome in healthy adults

Joo-Young Lee

Michigan State University

Kazem Kashefi

Michigan State University

Gemma Reguera (✉ reguera@msu.edu)

Michigan State University <https://orcid.org/0000-0003-4317-7933>

Research

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1 **Identification of a dynamic middle ear microbiome in healthy adults**

2 **Joo-Young Lee, Kazem Kashefi and Gemma Reguera***

3 *Department of Microbiology and Molecular Genetics, Michigan State University, MI,*

4 *USA*

7 *** Corresponding author:**

8 Gemma Reguera

9 567 Wilson Rd., Rm. 6190

10 Biomedical & Physical Sciences building

11 East Lansing, MI 48824

12 Tel.: (517) 884-5401

13 e-mail: reguera@msu.edu

14 ORCID: <https://orcid.org/0000-0003-4317-7933>

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Abstract

Background

The entrenched dogma of a sterile middle ear in health is incongruent with its periodic exposure to exhaled air when the tympanic (Eustachian) tube opens while swallowing, yawning or inhaling deeply. Tubal patency is brief but frequent to provide adequate aeration, equalize pressure across the eardrum and drain mucus down the sides of the oropharynx. It also provides a mechanism for aerial dispersal of microorganisms from the aerodigestive tract.

Method

We designed a pilot study and received institutional approval to collect otic secretions that naturally drain on the sides of the oropharynx, behind the palatopharyngeal arch. This protocol bypassed the need to surgically access the middle ear through or around the eardrum, allowed us to collect samples from individuals with no underlying otic conditions, and prevented sample cross-contamination. As controls, we also collected samples from the center of the oropharynx and buccal mucosae seeded by saliva, which may serve as sources of microbial dispersal into the middle ear.

Results

We sequenced 16S rRNA-V4 amplicons from otic, oropharyngeal and buccal samples collected from a cohort of 19 healthy young adults. The survey identified in the otic samples a diverse bacterial community with many oropharyngeal and buccal keystone taxa and most of the functional traits of the neighboring oral microbiomes. Neutral community models predicted a large contribution of oral dispersal to the composition of the otic microbiome as well as several taxa responsive to positive selection. This was further supported by the enrichment in the otic communities of obligate anaerobes of Bacteroidetes and Fusobacteria over facultative anaerobic Proteobacteria and Firmicutes. Furthermore, the prevalence of the anaerobic members

decreased with the more frequent otic aeration predicted from the equalization training of scuba divers recruited to the study.

Conclusions

These results challenge the long held view of a sterile middle ear and suggest instead that frequent seeding with oral microbes supports the establishment of a rich and robust otic community dynamically adapted to the episodic ventilation of the tympanic space.

Introduction

The notion that the healthy middle ear is sterile, like the entrenched dogma of lung sterility before [1], has been spread in the literature without strong scientific justification [2]. Perpetuating this tenet is the perception of the middle ear space as a fully isolated acoustic chamber. The eardrum does isolate the middle ear from the outer ear canal, preventing the immigration of microbes residing on the transcanal epithelium and providing selective control of gas permeation [3] (Fig. 1A). The major role of the tympanic membrane is however to transmit sound-induced vibrations to the hearing organ (inner ear's cochlea) via a delicate chain of small bones (ossicles) housed in the tympanic cavity [4] (Fig. 1A). A porous bone structure (mastoid antrum) with gas-filled, interconnected spaces communicates the cavity to the mastoid gas cell system to increase resonance and provide a medium for acoustic insulation and sound dissipation [5]. The tympanic cavity is also connected to the nasopharynx via the Eustachian tube (ET) (Fig. 1A). This tubal extension of the middle ear space is passively closed at rest to prevent interference from the noise of breathing and heartbeats [4]. Yet as air is consumed at the otic mucosa, negative pressure builds inside the tympanic cavity that contracts the eardrum and risks its integrity and functionality. Hence, the ET opens periodically (when we swallow, yawn or inhale deeply) to draw air in from the lower respiratory airways and equalize pressure across the eardrum [6]. Swallowing, for example, contracts the tensor veli palatini muscle that keeps the tube collapsed and elevates the

upper palate to seal the nasal cavity and communicate the tympanic cavity directly to the oropharynx [6]. Yawning or inhaling deeply can also exert positive pressure at the tube's nasopharyngeal orifice, increasing the differential pressure with the tympanic cavity and forcing the opening of the ET [6]. In addition, the otic epithelium secretes surfactants that lubricate the tubal walls and reduce the surface tension of the mucoid layer to facilitate patency [6]. Collectively, these mechanisms ensure that the tube dilates for only a short period of time (~400 milliseconds) to minimize noise interference [4] and permit high-frequency hearing [7]. Yet tube patency is frequent (approximately every minute when we swallow) to provide adequate ventilation and pressure relief [6].

ET patency is also critical to clear otic mucus and fluids that accumulate in the tympanic cavity as negative pressure builds. Mucociliary clearance facilitates the downward drainage of the secretions and, with them, foreign particles and microbes introduced with air [2]. Drainage is also facilitated by the pumping force exerted by the episodic contraction and extension of muscles around the tube [8]. High molecular weight glycoproteins (mucins) in the otic mucus confer additional protection, binding otopathogens and promoting their agglutination and opsonization [2]. The otic mucosa also contains proteins with antimicrobial activity or with motifs that recognize viral or bacterial surface carbohydrates to present them to macrophages and neutrophils for phagocytosis [9-12]. Anatomic barriers confer additional protection against microorganisms. To prevent the reflux of the mucus, the ET orifice emerges into the nasopharynx as an elevated cartilage covered by mucosa (torus tubarius) (Fig. 1B). This mucosal elevation is shaped like an inverted horseshoe to drain the otic secretions down the sides of the oropharynx (Fig. 1C). Additionally, the tympanic tube narrows down as it gets closer to the middle ear cavity [6] and grows longer [13], curves slightly [6] and increases its tilt [14] into adulthood to more effectively prevent the aspiration of mucus. The specialized anatomy of the ET and mechanisms for mucociliary and muscular clearance have been assumed to maintain the otic mucosa free of microbes in health [2]. Anatomical barriers, mucus drainage, and innate defenses are also active

processes of microbial elimination in the lower respiratory airways that were for long predicted to keep the lungs sterile [15]. Yet the micro-aspiration of saliva aerosols with air flowing through the oropharynx support rates of microbial immigrations that effectively counter-balanced the elimination rates to establish a lung microbiome [16]. A similar mechanism, but with exhaled air drawn from the lower respiratory airways, could seed the otic mucosa with oral migrants as well.

Several studies [17-20] have attempted to test if the otic mucosa of healthy individuals is indeed free of bacteria, albeit results thus far have been inconclusive. Its secluded location behind the eardrum motivated studies that collected samples as part of transcanal surgical procedures designed to treat a number of otic conditions. These interventions reached the middle ear cavity through or around the eardrum [21], limiting sampling to small mucosal areas of the cochlear promontory that can be reached without perturbing the ossicular chain (Fig. 1A). Two independent pediatric studies that surgically collected biopsy mucosal specimens from this region confirmed the presence of bacterial microcolonies in patients with a history of otitis media [17, 18] but only one study detected microbial cells in samples from healthy individuals [18]. Cross-contamination with microbes from the outer ear canal could not be ruled out either [18]. Non-invasive optical techniques also detected signals from bacterial biofilms in the middle ear of patients with a history of chronic ear infections but not in uninfected controls [22]. To improve the sensitivity of the detection method, Minami *et al.* [19] used transcanal surgery to collect middle ear mucosal swabs from pediatric and adult patients with or without a history of chronic ear infections. The detection in all the samples of a phylogenetically diverse pool of 16S rRNA amplicon sequences supported the conclusion that “the human middle ear is inhabited by more diverse microbial communities than was previously thought” [19]. However, the study did not include controls from the outer ear canal, which a more recent study showed to contaminate the swabs during sampling [20]. As a result, the otic samples were enriched (85%) in Proteobacteria and Actinobacteria [19], which are also the most abundant phyla in the outer ear canal [23, 24]. Transcanal sample collection in these prior studies [17-20] also considered as healthy controls, individuals undergoing surgery to

121 treat otic conditions (e.g., otosclerosis, middle ear malformation, Bell's palsy and deafness) that
122 are associated with local inflammation and viral and bacterial infections [25-28]. Hence, the
123 assumption that these individuals are healthy is questionable.

124 To bypass limitations of previous studies, we sought to extract and sequence DNA from
125 the otic secretions that naturally drain laterally behind the palatopharyngeal arch (Fig. 1C). We
126 therefore designed a pilot study and received institutional approval to collect otic samples from a
127 cohort of 19 healthy young adults. Some of the participants ($n = 9$) were certified scuba divers
128 and, thus, had training in equalization techniques that control the aperture of the ET and promote
129 middle ear ventilation [6]. We, therefore, used these individuals as internal controls for middle ear
130 aeration. To assess the contribution of oral migration to the middle ear, we also collected samples
131 from buccal mucosae (inner lining of the cheeks, upper gums and palate) that best represent the
132 flexible and keratinized epithelia of the oral cavity that seed the salivary microbiome [29].
133 Additionally, we collected samples from the center of the oropharynx, which we hypothesized to
134 be the primary source of saliva aerosols introduced into the middle ear. Amplicon sequencing of
135 the 16S rDNA V4 region identified in the otic secretions a diverse and robust microbiome of
136 predominantly anaerobic taxa and revealed changes in community structure that correlated well
137 with the frequency of otic aeration predicted for the participants. Yet all of the subjects shared a
138 core otic microbiome taxonomically and metabolically similar to the oropharyngeal and buccal
139 communities, albeit substantially different from the nasal microbiome. Our results challenge the
140 long held view of a sterile otic mucosa and suggest instead that the middle ear is a dynamic
141 ecosystem seeded by oral microbes and enriched in organisms more suited for growth under
142 episodic aeration.

144 **Results**

145 **Sequencing of a diverse and robust otic microbiome in healthy, young adults.**

Using an institutionally-approved protocol, we collected otic, oropharyngeal and buccal samples from 19 healthy young adults (11 women, 8 men) approximately 20 years old on average (Supplementary Table 1, Additional File 1). All of the participants reported no recent history of respiratory infections and/or antibiotic treatment and passed an onsite physical exam that ruled out nasal congestion/dripping as well as inflammation and abnormalities of the nose, mouth, and ears. Among the participants were 9 certified scuba divers who reported similar training in middle ear equalization techniques and diving experience at depths of >60 ft (Supplementary Table 2, Additional File 1). The diver cohort allowed us to investigate adaptive responses of the resident microbial communities to the frequency of aeration of the tympanic cavity. Subjects meeting the eligibility criteria and passing the onsite physical exam first rinsed their mouths with sterile saline to remove food debris and performed a series of equalization exercises (deep inhaling, yawning and swallowing) that maximized otic drainage down the sides of the oropharynx. The team's physician used a tongue depressor to improve spatial access to the back of the throat and introduced a sterile flocked swab to gently rub in an ascending motion the left and right mucosal channel of the torus tubarius, behind the palatopharyngeal arch (Fig. 1B-C). The physician then used separate swabs to collect samples from the center of the oropharynx and buccal mucosae (inner lining of the cheeks, upper gums and palate).

Illumina sequencing of 16S-V4 amplicons from all the samples yielded a total of 12,219,721 reads, with an average number of 214,381 ($\pm 8,132$) reads for each region sampled per participant. To normalize differences in read number and therefore diversity among the samples, we rarified the sequences to a depth of 2,313 reads per sample prior to assigning operational taxonomic units (OTUs). We identified in the otic samples an average of 95 (± 26) genus-level OTUs compared to 100 (± 28) and 113 (± 20) in the center of the oropharynx and the buccal samples, respectively. Thus, genus-level diversity in the otic samples was within the levels obtained for the richly colonized mucosae of the oral cavity [29].

Alpha diversity analyses based on observed species, Shannon diversity and Simpson evenness provided additional evidence for the presence of a diverse and robust otic community (Fig. 2A). The otic and oropharyngeal communities were similar in terms of alpha diversity but they tended to have lower number of observed species than in the buccal samples. The trend reversed when considering Shannon diversity and Simpson evenness, which were lower in the buccal communities compared to the other collection sites. The most notable differences were for Simpson's evenness between otic and buccal samples, which showed the greatest distribution of taxa in the otic communities ($p < 0.001$). Estimation plots of the standardized mean differences (Δ) among alpha diversity indices per site also showed the most significant differences for Simpson's evenness, which followed the trend otic>oropharyngeal>buccal (Fig. 2A). Thus, despite having similar, if not lower, number of species, the otic communities were more even than the oropharyngeal and buccal communities, suggestive of a microbial population that has the robustness and adaptability needed to function in a fluctuating environment [30].

Beta diversity analyses highlighted similarities in the phylogenetic distribution of the otic and neighboring communities that agree well with a model of oral migration in the middle ear (Fig. 2B). Thus, Principal Coordinates Analysis (PCoA) transformation plots of weighted UniFrac distances showed site-specific clustering of otic and buccal samples with some overlap in PCoA space along the variance obtained for the first axis (Fig. 2B). By contrast, the oropharyngeal sequences spread across the two axes to overlap with both the otic and buccal clusters. This is consistent with the oropharynx serving as central hub for microbial immigration from the oral cavity to the middle ear. Indeed, the oropharynx is seeded with saliva and air, and also provides the saliva aerosols that are introduced into the middle during the patency of the ET. This shows as a substantial spatial overlap between the oropharyngeal and the otic and buccal communities in the PCoA ordination graphs (Fig. 2B).

Evidence for dynamic adaptations of the otic communities to episodic aeration.

Taxonomic analyses of the OTUs assigned to the amplicon sequences revealed an otic community with a phyla structure more similar to the oropharyngeal than to the buccal communities (Fig. 3A). Bacteroidetes, Fusobacteria, Proteobacteria, and Firmicutes collectively accounted for >95% of the OTUs identified at the three sites. However, the relative abundance of the strictly anaerobic phyla increased in the middle ear and oropharynx compared to the more aerated buccal communities. For example, the obligate anaerobic Bacteroides and Fusobacteria phyla were more prevalent in the otic and oropharyngeal (55% and 48%, respectively) than in the buccal communities (24%) (Fig. 3A; Supplementary Table 3, Additional File 1). The lower representation of these anaerobic phyla in the buccal samples agrees well with the more frequent aeration of the oral cavity compared to the oropharynx and the middle ear. Indeed, the buccal communities enriched for facultative anaerobes and aerobic taxa in the Proteobacteria and Firmicutes (72% of all the phylotypes), but the abundance of these buccal phylotypes decreased progressively in the less aerated mucosa of the oropharynx and middle ear (Fig. 3A). Thus, the prevalence of the dominant phyla matched well with the frequency of aeration (therefore, oxygen availability) at each body site.

We next assessed the contribution of aeration frequency to community assembly by reconstructing the structure of the otic and oral communities for the recreational divers recruited to the study. Consistent with increased otic ventilation in this cohort [31], the relative abundance of otic Bacteroidetes and Fusobacteria decreased ($p = 0.02$) from 62% in non-divers ($n=10$) to 48% in divers ($n = 9$) (Supplementary Table 3, Additional File 1). We correlated the changes in Bacteroidetes abundance with fluctuations of two genera (*Prevotella* and *Alloprevotella*) (Supplementary Fig. 1, Additional File 1) and ruled out gender effects (Supplementary Fig. 2, Additional File 1). The fact that all participants had a similar representation of Bacteroidetes phylotypes in the oropharyngeal communities that could serve as sources of microbial immigration also made dispersal effects unlikely (Fig. 3B). Rather, the representation of Bacteroidetes in the

otic communities is consistent with an adaptive response to aeration frequency in the middle ear. Divers also had a lower otic representation of *Fusobacteria* (Fig. 3B) that matched well with decreases in the relative abundance of the genus *Fusobacterium* (Supplementary Fig. 1, Additional File 1). However, these phylotypes are also less abundant in the oropharyngeal and buccal communities of the diver's cohort (Fig. 3B). This suggests that the representation of *Fusobacteria* in the otic microbiome is conditioned by the abundance of this phylum in the oral communities that serve as sources of dispersal. Dispersal effects could have also contributed to the overrepresentation in divers of otic Firmicutes, particularly the genera *Streptococcus* and *Veillonella*, which are more abundant in the buccal source communities of this cohort of participants (Supplementary Fig. 3, Additional File 1). Therefore, the taxonomic composition of the otic communities is the result of neutral processes (dispersal from the oral sources and ecological drift in the middle ear) as well as positive selection of groups better suited for growth and reproduction under episodic aeration.

The oral cavity as a seeding source for the otic communities.

We gained insights into the contribution of oral bacteria to the seeding of the middle ear by defining the core microbiomes at each collection site and the degree of shared membership among the core communities (Fig. 4). We included in these analyses genera represented in at least half of the participants within each group (non-divers and divers) and used this information to identify core members for each collection site (Fig. 4A). The core otic diversity comprised 76 genera and included 66 taxa from the oropharyngeal and buccal core communities (Fig. 4B). Thus, 87% of the otic genus diversity was shared with the neighboring oral communities. The remaining 13% consisted of low abundant OTUs (<0.1%) that were present in one or two sites only (Additional File 2). Transience could explain the detection of some of these low abundant taxa in the otic samples. We detected, for example, *Massilia*, an aerobic genus that is ubiquitous in soils and enters and disperses through the aerodigestive tract via aerosols [32]. Also among the rare

taxa was *Peptococcus*, a Firmicutes genus within the Gram positive anaerobic cocci (GPAC) group that enters the aerodigestive tract via saliva or saliva aerosols [33]. Similarly, some of the otic OTUs were assigned to the *Eubacterium brachy* group, within the Firmicutes, and to uncultured *Leptotrichiaceae* within the Fusobacteria, which are groups of obligate anaerobes known to disperse through the aerodigestive tract as well [34, 35].

We next implemented a neutral community assembly model (adapted from Sloan *et al.* [36] by Venkataraman *et al.* [37]) to predict the contribution of unbiased (neutral) processes to the otic core composition (Fig. 4C). The goodness-of-fit (R^2) values were similar when considering the oropharynx ($R^2 = 0.37$) and buccal ($R^2 = 0.44$) samples as source communities. This coefficient of determination, though modest (0, no fit; 1, perfect fit), could explain the presence of at least 70% of otic OTUs as the result of dispersal from the oropharyngeal and buccal communities and ecological drift once in the middle ear environment. Most of the remaining OTUs fell above the confidence interval and, therefore, represent taxa over-represented in the otic communities compared to the two sources considered (green symbols in Fig. 4C). More than half of these OTUs (38 in all) deviated positively from the neutral model with both the oropharyngeal and buccal source communities (Additional File 3). These taxa are predicted to have a competitive advantage in the middle ear, either because they are better suited to grow in the otic mucosa and/or have a greater dispersal ability relative to other members of the source communities. The fact that most of these OTUs are rare oral taxa enriched in the otic samples (Supplementary Fig. 4, Additional File 1) suggests, however, that they are not dispersal-limited but rather have a growth advantage in the middle ear microenvironment.

The central location of the oropharynx in the aerodigestive system permits the seeding of its mucosa with saliva and the micro-aspiration of saliva aerosols into the middle ear. Therefore, we expected the oropharynx to contribute to the seeding of the middle ear more strongly than the buccal communities. To test this, we compared the mean relative abundance of the 66 genera that are shared by the three core communities (Supplementary Fig. 5, Additional File 1). These

analyses revealed a stronger positive association of otic taxa abundance with the oropharyngeal (Pearson's correlation coefficient, $R^2=0.97$) than with the buccal ($R^2=0.62$) source communities, consistent with the oropharynx serving as primary source for microbial immigration to the middle ear. The most significant deviations (at least two-fold increases or decreases in otic abundance compared to the oropharyngeal source) were for rare taxa with mean relative abundance $<0.03\%$ (Supplementary Fig. 5, Additional File 1). Five of these rare OTUs (*Treponema* 2, *Corynebacterium*, *Porphyromonas*, *Parvimonas*, and *Stenotrophomonas*) were over-represented in the middle ear while the other 8 (*Acidibacter*, *Corynebacterium* 1, *Ralstonia*, *Catonella*, *Rothia*, *Eubacterium yurii* group, and other groups) were under-represented. These positive and negative deviations from the linear correlation of OTU abundance were exacerbated when considering the buccal communities (Supplementary Fig. 5, Additional File 1), which are more distant sources of microbial immigration.

Analyses of the abundance patterns among the dominant genera also provided evidence for the oropharynx serving as central hub for the dispersal of oral migrants into the middle ear. For these analyses, we normalized taxa abundances per individual using the z-score method to generate a heatmap of the most abundant genera shared by the three collection sites (Fig. 4D). We identified the same 20 dominant genera in all the samples, but the average abundance of each taxon changed with the collection site (Fig. 4E). The representation of the otic genera more closely mirrored the oropharyngeal than the buccal communities, supporting the notion that the oropharynx is a primary source for microbial immigration into the middle ear. We also observed abundance trends that correlated well with the selection of obligate anaerobes within the Bacteroidetes (family *Prevotellaceae*) and Fusobacteria (*Fusobacterium* and *Leptotrichia* spp.) phyla in the otic communities. *Veillonella*, an anaerobic genus of oral Firmicutes known to co-aggregate and metabolically cooperate with *Streptococcus* in dental plaque [38], was also abundant in the otic communities. By contrast, facultative anaerobic Proteobacteria (*Haemophilus* spp.) and Firmicutes (*Streptococcus* spp.) were most prevalent in the buccal samples. *Neisseria*

OTUs provided an example of a genus that is similarly represented in the three locations and, therefore, neutrally distributed. Indeed, this is a proteobacterial group of oropharyngeal and oral commensals that grows best aerobically [39] yet can disperse into the lower respiratory airways and lungs via saliva aerosols [40]. A similar mechanism of dispersal could explain the transience of these commensals in the middle ear.

Similar functional structure of the otic and oral microbiomes.

Given the similarities in community membership among the three collection sites, we predicted a high degree of functional redundancy among their members as well. To test this, we used metabolic inference methods to predict the metabolic structure of the otic microbiome and describe relationships with the spatially close communities of the oropharynx and buccal mucosae. A heatmap of Z-score transformed relative proportion of functions represented at each collection site (1% cutoff) revealed a high degree of redundancy in core functions (Supplementary Fig. 6, Additional File 1). These core functions are stable in non-divers and divers (Fig. 5A) and have a high representation of membrane transport functions and modules associated with the metabolism of amino acid and carbohydrates (Supplementary Fig. 6, Additional File 1). Such metabolic functions often prevail in mucosal-associated communities that rely on proteins and mucin glycoproteins secreted in the mucosa as sources of amino acids and carbohydrates to support microbial growth [41]. Also with high representation in the three microbiomes were functions for genetic processing and information (replication and repair and, to a lesser extent, translation) that are critical to support cell growth (Supplementary Fig. 6, Additional File 1). By contrast, cell motility functions were low. This is not unexpected given the primary contribution of passive mechanisms of dispersal (i.e., via saliva and/or saliva aerosols) to microbial immigration in these body sites.

Despite the overall similarity in core functions at the three collection sites, we identified in pairwise comparisons some significant changes in the otic communities compared to the

oropharyngeal and buccal sources (Fig. 5B). The most notable differences were the lower representation of membrane transport functions yet higher relative proportion of amino acid and energy metabolism modules in the otic and oropharyngeal communities compared to the buccal communities. These differences likely reflect quantitative and qualitative changes in the nutrients that are available to support the growth of the resident microorganisms. Dietary substrates are, for example, abundant in the mouth yet less accessible in the oropharynx due to disturbance by the frequent cycles of air inhalation and exhalation. Dietary nutrients are also scarce in the middle ear due to the limited carriage of external nutrients in saliva aerosols. The oropharyngeal and, even more so, the otic communities are, therefore, predicted to sustain their trophic webs with host-derived nutrients such as proteins and mucin glycoproteins secreted to the mucosa [41]. This helps explain why the relative proportion of membrane transport functions decreased while modules for amino acid and energy metabolism more represented in the otic and oropharyngeal communities.

Discussion

The identification of a diverse and robust microbiome in otic samples collected from healthy young adults challenges the entrenched view of a sterile middle ear mucosa and suggests instead that microorganisms colonize the otic mucosa and establish a site-specific community adapted to episodic ventilation. We initially reasoned that the frequent openings of the ET would enable the micro-aspiration of saliva aerosols from the oropharynx and the seeding of the otic mucosa with oral migrants. As in other regions of the upper respiratory tract [42], mucociliary activity promotes the clearance of the otic mucus and, with it, bacteria residing the mucosa. The periodic contraction and expansion of muscles around the ET also contributes to the clearance of mucus and fluids from the tympanic cavity and their drainage on the sides of the oropharynx [8]. Non-invasive sampling of these secretions permitted the amplification of 16S-V4 rRNA bacterial

sequences and the identification of an otic community with genus-level diversity comparable to the neighboring oral communities, which are among the richest and most diverse in the human body [29]. PCoA plots revealed the spatial clustering of the otic communities expected for site-specific populations and overlap with the oropharyngeal and, to a lesser extent, buccal communities that we predicted to serve as seeding sources (Fig. 2B). The taxonomic composition of the otic communities was also more similar to oral than nasal microbiomes, despite the closer proximity of the ET orifice to the nasopharynx (Fig. 1B). Actinobacteria, for example, is the most abundant nasal phylum [43] but only accounted for ~1% of the otic OTUs (Supplementary Table 3, Additional File 1). Instead, the otic microbiome enriched for Bacteroidetes (38±6%), a strictly anaerobic phylum with lower representation (<10%) in the more aerated mucosae of the nasal cavity [43]. The relative abundance of Firmicutes in the otic samples (21±4%) was within the ranges reported in the nasal microbiome [43] but while nasal Firmicutes are dominated by *Staphylococcus* [44], this genus was not significantly represented in the otic samples (Fig. 4). Rather, otic Firmicutes were dominated by *Streptococcus* and, to a lesser extent, *Veillonella*, which are two of the most abundant genera in the oral cavity (Fig. 4). The otic communities also shared most of the keystone taxa of the core oral communities that we hypothesized would serve as sources of dispersal (Fig. 4). Indeed, the three mucosal sites sampled in this study harbored microbial communities dominated by the same 10 genera (*Prevotella* 7, *Fusobacterium*, *Prevotella*, *Veillonella*, *Haemophilus*, *Leptotrichia*, *Alloprevotella*, *Streptococcus*, *Neisseria* and *Porphyromonas*) (Fig. 4). These genera represented four phyla (Bacteroidetes, Fusobacteria, Proteobacteria and Firmicutes) that collectively accounted for >90% of all the OTUs at each collection site. However, the relative abundance of anaerobic taxa (Bacteroidetes and Fusobacterial genera) was higher in the middle ear than in the neighboring oral mucosae, which selected instead for facultative aerobes in the Proteobacteria (*Haemophilus*) and Firmicutes (*Streptococcus*) (Fig. 4).

Alpha diversity analyses (Fig. 2A) provided additional evidence for the presence of an otic community as rich and diverse as the oral communities yet adapted to disturbances. The analyses revealed, for example, increases in species evenness in the otic communities that are often associated with microbiomes having the robustness and functional stability needed to adapt to environmental fluxes [30]. Redox fluctuations are expected to be common in the middle ear, due to the periodic pulses of air that enter the middle ear cavity when the ET opens. Episodic exposure of the otic mucosa to air provides a reasonable explanation for the enrichment in the otic communities of strict anaerobes, particularly those in the Bacteroidetes phylum (Fig. 3). The brief cycles of tubal dilation limit the volume of air entering the tympanic cavity to 4-5 μ l [6]. Otic ventilation also uses exhaled air [6], which has a lower concentration of oxygen than atmospheric air. Importantly, swallowing opens the ET once every minute during the wake hours but patency slows down (every 5 minutes) during sleep [6]. This suggests that conditions of oxygen limitation prevail in the middle ear. Aerotolerant strains will have an adaptive advantage under these conditions and would be key to preserving community stability. The most prevalent otic Bacteroidetes were members of the family *Prevotellaceae*, which despite their strictly anaerobic metabolism can adaptively evolve oxygen tolerance under selective pressure [45]. By contrast, obligate anaerobes lacking adaptive strategies against redox fluctuations will be outcompeted. This could explain why *Fusobacterium* was more abundant in the otic microbiome of non-divers, a group that lacks the equalization training and diving experience associated with increased otic ventilation.

Nutritional variables cannot be excluded as selective forces in the middle ear either. We sequenced, for example, twice more Bacteroidetes phylotypes in the otic samples than typically found in the strictly anaerobic environment of the colon (~16%) [46]. The representation of Bacteroidetes in the gut is influenced by the presence of dietary complex carbohydrates, which *Bacteroides* species break down to provide fermentable sugars for Firmicutes [46]. Nutrient availability in the middle ear is however limited to host-derived nutrients such as lipids, proteins

and mucin glycoproteins secreted by the mucosal epithelium. Otic mucins contain 39.5% protein and 60.5% carbohydrate (N-acetylglucosamine, galactose, N-acetylgalactosamine, fucose and sialic acid) [41]. Bacteroidetes are well equipped to break down the mucin [47] and could release the simple sugars to support the growth of fermentative bacteria in the otic mucosa as well. Gut Bacteroidetes play similar roles in mucin and fiber degradation and release sugars that strict anaerobes in the class Clostridia (95% of all the gut Firmicutes) [46] can ferment. This class was however under-represented (~4%) in the otic communities. Instead, otic Firmicutes were represented by *Streptococcus* and *Veillonella*, abundant oral taxa that form metabolically linked co-aggregates during the primary colonization of the tooth surface [38]. The metabolic co-dependence of these two bacteria is established with the fermentation of sugars to lactate by the *Streptococcus* partner and the fermentation of lactate by *Veillonella* to produce propionate, acetate, CO₂ and H₂ [48, 49]. A similar syntrophic consortium could promote the fermentation of mucin sugars in the otic communities and the production of short chain fatty acids critical to mucosal health. The lactate dependency of *Veillonella* may also permit syntrophic interactions with Bacteroidetes partners that ferment simple sugars into lactate [50]. The high representation of pathways for carbohydrate and amino acid metabolism and reduced membrane transport predicted for the otic phylotypes (Fig. 5) does indeed support a trophic web in the otic mucosa driven by the metabolism of mucins.

The similarities between the otic and gut trophic webs are not unexpected given the fact that both mucosae are seeded with oral microbes and both sites enrich for anaerobic migrants. A wide range of microbes enter the mouth with air, food, and via host-to-host contact before dispersing into the aerodigestive tract via air and/or saliva [51]. The oral cavity provides a heterogenous landscape (tooth surfaces, tongue, gingival crevices, palate, etc.) that locally selects for the growth of specific taxa, increasing their representation in saliva and their dispersal potential to other parts of the gastrointestinal tract and the lower respiratory airways [40]. Neutral models of community assembly suggest that the majority of microbial sequences recovered from

the upper gastrointestinal tract and the lungs in healthy individuals disperse from oral reservoirs [37]. Similarly, oral dispersal had a profound influence in shaping the composition of the otic microbiome. Thus, we identified in the oropharyngeal and buccal communities most of the core otic taxa (87% of the otic OTUs) (Fig. 4B), including many taxa known to disperse through the aerodigestive tract with air and/or saliva. Further, most (~70%) of the shared OTUs were neutrally distributed in a neutral model fit when considering the oropharynx or buccal communities as potential sources of immigration (Fig. 4C). ET patency only lasts about 400 milliseconds but occurs frequently (about 1,000 times a day just by swallowing) [52], providing a constant source of oral migrants. The process is analogous to the subclinical micro-aspiration of saliva aerosols that seeds the lower airways and the healthy lungs with oral microbes [42]. But unlike the seeding of the lungs with saliva aerosols carried during inhalation, microbial immigration into the middle ear is via exhaled air drawn from the lungs during ET patency. Not surprisingly, oral microbes commonly carried into the lower respiratory tract via aerosols such as *Prevotella*, *Veillonella*, *Streptococcus*, and *Fusobacterium* [42] were also among the most abundant in the otic microbiome (Fig. 4). The adapted insular model of the lung microbiome [16] based on island biogeography theory [53] explains the constant presence of these microbes and their abundance in the lower airways as an equilibrium between the rates of microbial immigration (primarily by micro-aspiration during inhalation) and extinction (exhalation, coughing, etc.) with little contribution from the local growth of its members [16]. A similar model could explain the colonization of the middle ear with oral seeds, though with some notable differences. The middle ear is but a few centimeters away from the richly colonized mucosa of the oropharynx (the lungs are about half a meter), which could significantly increase the rates of microbial immigration. Spatial proximity could also permit the direct migration of oropharyngeal bacteria by swarming, a mode of flagellar motility that is stimulated by mucosal lubricants such as surfactants and mucin [54, 55]. Extinction rates in the middle ear would be determined by the efficiency of mucociliary and muscular clearance [8, 56]. The enrichment of specific groups in the otic samples suggests

that these mechanism for mucus clearance cannot prevent the colonization of the otic mucosa by oral migrants. Furthermore, most of the otic OTUs that deviated from the neutral model were over-represented in the otic communities compared to the oropharyngeal or buccal sources (Fig. 4C), suggesting a process of positive selection. Among the oral sequences enriched in the otic environment were strict anaerobes within the Bacteroidetes, Fusobacteria and Firmicutes (Additional File 3). Also over-represented were many Proteobacterial sequences assigned to groups of facultative anaerobes, which could have a competitive advantage for growth with intermittent aeration. Indeed, otic Proteobacteria represented 16% of the sequences identified in the non-divers otic samples and had an even higher representation (~25%) in the more aerated ears of divers. By contrast, many oral Proteobacteria are negatively selected under the strictly anaerobic conditions of the colon, reducing their representation to ~0.1% of the gut phylotypes [46]. Dispersal ability could have also contributed to the over-representation of some oral taxa in the otic communities. More frequent otic ventilation, as predicted for divers, could also increase the rates of micro-aspiration of saliva aerosols from the oropharynx and the dispersal of oral migrants. This could explain the higher representation in divers of facultative anaerobic taxa such as *Streptococcus*, a genus that is most abundant in the buccal communities (Fig. 4).

Our study suggests that the structure of the otic communities is responsive to aeration. Several host variables could have also contributed to these responses. Scuba diving can cause small changes in pulmonary function that could increase the rates of aerial dispersal into the middle ear [31]. Anatomy and even body position and posture also influence ET function [6]. Thus, ET opening time is shorter when lying down because the increased blood flow to the head and neck causes venous engorgement around the tympanic tube [57, 58]. As a result, the mean volume of air passing through the tube's lumen is two thirds lower when in the supine than the prone position [59]. Thus, we cannot rule out that increased otic ventilation in divers resulted from higher levels of physical activity in this group. Further, subtle differences in the anatomy of the ET are not uncommon [6] and could have affected the rates of microbial immigration over extinction

and, by extent, the structure of the otic community. Importantly, we selected a homogenous cohort of young adults among the college population to minimize diet effects but we cannot exclude that dietary preferences contributed to intra- or inter-group differences. Future investigations could address these variables in larger surveys that select participants based on their dietary and exercise routines. Also important for future studies are insights into the intrinsic and extrinsic factors that lead to the positive or negative selection of taxa in the middle ear, particularly otopathogens. Our study highlighted correlations that point at a critical role for aeration and host-derived nutrients in the assembly of the otic communities, but other variables such as pressure fluctuations and sound-induced vibrations could exert selective pressure and permit the diversification of oral taxa into otic lineages better suited for growth and reproduction in the middle ear mucosa. This, in turn, is expected to influence the functionality of the otic community, its interactions with the host mucosa and the outcome of infections.

Conclusions

The notion that the healthy middle ear is sterile is incongruent with its exposure to saliva aerosols, which are introduced every time we swallow and the tubal extension of the tympanic cavity (the ET) opens. Here, we described the non-invasive collection of otic secretions drained on the sides of the oropharynx with the cycles of tubal aperture and the sequencing of a diverse and robust otic microbiome with many of the keystone taxa and functional attributes of the neighboring oral communities. Our results challenge the long held view of a sterile otic mucosa in health and suggest instead that the middle ear is a dynamic ecosystem seeded by oral microbes and enriched in organisms better suited for growth under episodic aeration. Furthermore, we describe physiological attributes of the colonizers of the middle ear that support a critical role for the otic microbiome in mucosal health. This knowledge may be considered when designing prophylactic treatments against otic infections and disfunctions.

Methods

Participants' recruitment and sampling. As part of a larger study to investigate the middle ear microbiome, we recruited healthy adult individuals by advertisement at the Michigan State University campus and diving shops in the state of Michigan from September to November of 2017. The study was approved by Michigan State University's Institutional Review Board (IRB #17-502) and all subjects gave signed informed consent to participate. We selected 19 participants (10 non-divers [5 women and 5 men] and 9 recreational divers [6 women and 3 men] that met the eligibility criteria of age and health to provide the DNA samples for amplicon sequencing. Participants age was between 18 and 32 years old, with an average of ~20 (Supplementary Table 1, Additional File 1). The participants were scheduled for sample collection on November 18th of 2018 in a clinic room at the Clinical and Translational Sciences Institute on the Michigan State campus. Each volunteer filled out a questionnaire to provide relevant information (age, gender, race, medical history with a focus on ear, nose, and mouth conditions and past antibiotic use; recreational/professional aquatic activities). Exclusion criteria were pregnancy, chronic health problems, asthma, persistent allergies, and recent (less than 3 months) antibiotic treatment or conditions affecting the ear, nose and throat (e.g., bronchitis, strep throat, ear infections, hearing loss, problems with balance, etc.). Divers were also asked to show proof of current scuba diving certification and provide information about their diving training (Supplementary Table 2, Additional File 1). A physician specifically trained for the task interviewed each participant in the presence of the team leader and prior to sample collection to ensure they understood the study protocol and to rule out mental incapacity and/or cognitive impairment for consent. The physician then performed a general otolaryngologic examination (otoscopic examination of the ear, nose, and mouth) to rule out inflammation or other conditions affecting participant's eligibility. The results of the otoscopic exam were recorded in an examination sheet.

The participants were asked to rinse their mouth with a sterile saline solution to remove food debris and to follow a series of deep inhalation and yawn cycles that forced the opening the tympanic tube (they had to hear a “pop”) and the drainage of the middle ear fluids. They were then asked to swallow to naturally open the ET one more time and to open their mouth to initiate sample collection. We used a sterile tongue depressor to improve access to the back of the mouth and prevent oral and/or tonsil contamination. Sample collection was with FLOQSwabs™ (Copan) and storage was in collection tubes filled with eNAT™ medium (Copan). We first collected in a single swab the left and right otic secretions, using an ascending motion to swab the mucosal channels that laterally drain the otic fluids behind the palatopharyngeal arch. The physician then used separate swabs to collect control samples from the central region of the oropharynx and from the inner lining of the cheeks and upper gingiva and palate (buccal samples). To preserve the anonymity of the participants, we barcoded the swab samples and all the forms and questionnaire collected for each individual. All samples were stored in the collection tubes at 4°C for 8-24 h before transport to the lab for immediate processing.

DNA extraction and 16S rRNA amplicon sequencing. We vortexed the transport tubes at medium speed for 1 min to detach the specimens and used a 400-μl aliquot of the cell suspension for DNA extraction with the FastDNA™ Spin kit (MP Biomedicals). The protocol for DNA extraction followed manufacturer’s recommendations, except that cell lysis used 800 μl of cell lysis buffer instead of the recommended 1 ml. Sample homogenization used a Mini-Beadbeater (BioSpec Products) operated at maximum speed for 40 seconds and followed three cycles of mechanical homogenization interspersed with sample cooling on ice for 1 min. DNA quantification in the samples was with a NanoDop™ (Thermo Fisher Scientific) and a Qubit™ dsDNA HS assay (Thermo Fisher Scientific). The quality of the extracted DNA for amplification of the V4 region of the 16S rRNA genes was tested using dual indexed Illumina compatible primers (515f [GTGCCAGCMGCCGCGGTAA] and 806r [GGACTACHVGGGTWTCTAAT]) [60] in a 25-μl

PCR reaction containing 12.5 µl 2xGoTaq® Green Master Mix (Promega), 0.1 µM of each primer, 0.2-20 ng of DNA template, and nuclease-free water. The PCR amplification also included controls that replace the DNA template with the elution buffer used in DNA extraction, sterilized water and eNAT transport medium. Visualization of PCR products was on 1.2% agarose gels. Library preparation and amplicon sequencing were performed by the Genomics Core staff at Michigan State University's Research Technology Support Facility (RTSF) and followed standard protocols for PCR-amplification of the V4 hypervariable region of the 16S rRNA gene using the 515f/806r primer pair, normalization of the PCR products in SequalPrep DNA Normalization plates (Thermo Fisher Scientific), and cleaning of the pooled samples with AMPureXP magnetic SPRI beads (Beckman Coulter). Quality control and DNA amplicon quantification used the Qubit™ dsDNA HS assay, LabChip® GX DNA HS assay (PerkinElmer), and Kapa Library Quantification kit for Illumina platforms (Kapa Biosystems). Sequencing of the pooled amplicons was on an Illumina MiSeq v2 standard flow cell using a 500 cycle v2 reagent cartridge for 250 bp paired-end reads and used standard Illumina quality control steps, including base calling by Illumina Real Time Analysis (RTA) v1.18.54, demultiplexing, adaptor and barcode removal and RTA output conversion to FastQ format with Illumina Bcl2fastq v2.19.1.

Sequence data processing and analysis. We used USEARCH (v10.0.240) [61] to process the paired-end reads (FASTQ files) and merge paired-end sequences, quality-filter, dereplicate, remove singletons, pick OTUs and match them against the Silva database. Briefly, processing of raw reads and quality filtering used the UPARSE pipeline [62] and clustered into operational taxonomic units (OTUs) at 97% identity against the Silva Database (v. 1.19) [63] using previously outlined protocols [64, 65]. Reads without a database match were clustered in *de novo* mode at 97% identity [63]. Taxonomic assignment and diversity analyses used the QIIME workflow [66]. A summary of the script used in this study is available on Github (https://github.com/mutantjoo0/RegueraLab_ONR).

Intra-group (alpha) and inter-group (beta) diversity analyses used the functions available in the QIIME pipeline [63]. Alpha diversity analyses measured in each sample the species richness (number of observed species), their abundance and evenness (Shannon diversity index, H) and evenness (Simpson). Beta diversity analyses applied the weighted UniFrac metrics [67] to calculate pairwise phylogenetic distances between sets of sequences and generate a distance matrix for Principal Coordinates Analysis (PCoA). MS Excel 2016 was then used to visualize the inter-group relatedness in PCoA plots and calculate the statistical significance of the UniFrac distance between the otic and control samples with the t -test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). InteractiVenn [68] was used to generate Venn diagrams and identify the core microbiome (Fig. 4A). When indicated, we applied estimation statistics (www.estimationstats.com) to assess the effect of size distribution and generate two-group estimation plots, as described elsewhere [69, 70]. The estimation plots show the mean difference (Δ), the bootstrapped distribution of the mean difference and the bootstrapped 95% confidence interval of mean difference.

We analyzed the 16S-V4 phylogenetic data with the PICRUST software package [71] to predict functional traits based on the Kyoto Encyclopedia of Genes and Genomes Orthology (KO) classification [72] on the Nephela cloud platform [73]. Visualization of the taxonomic and functional profiles for each sample used heatmaps generated with the Heml toolkit [74]. To illustrate the normalized distribution across the values, we used row Z-score normalized relative abundances and relative proportions or applied the average linkage clustering method and Euclidean distance metric to calculate pair wise distance and similarity for hierarchical clustering in heatmaps. The neutral community model applied in this study was adapted from Sloan *et al.* [36] using custom R scripts developed by Venkataraman *et al.* [37]. Briefly, the analyses calculated OTU abundance in the source (no. of OTU sequences with OTU in source community/total no. of sequences in source community) and the frequency of detection of each OTU in the otic communities (no. of participants with that OTU detected in the otic sample/total no. of individuals surveyed). A beta probability distribution was then used to predict the frequency

of detection of each OTU in the otic samples as a result of neutral processes (dispersal and ecological drift) [36]. After optimization of the fitting parameter using a least-squares approach, we calculated the 95% binomial proportion confidence intervals for the neutral model and the goodness-of-fit coefficient of determination (R^2), which ranges from 0 (no fit) to 1 (perfect fit). OTUs that fall outside the upper or lower confidence intervals are not neutrally distributed and identify OTUs likely to undergo positive or negative selection in the target community, respectively.

Availability of data and materials

Raw sequence reads have been deposited in the NCBI Sequence Read Archive under BioProject ID: [PRJNA473788](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA473788).

List of abbreviations

ET: Eustachian tube

OTUs: Operational taxonomic units

PCoA: Principle coordinates analysis

PCR: Polymerase chain reaction

rRNA: Ribosomal RNA

Declarations

Ethics approval and consent to participate

The research proposed in this study was approved on May 17 of 2017 by the Institutional Review Board (IRB) at Michigan State University, East Lansing, Michigan, United States of America. The committee found the research project to be appropriate in design, to protect the rights and welfare of human subjects, and to meet the requirements of Michigan State University's Federal Wide Assurance and the Federal Guidelines (45 CFR 46 and 21 CFR Part 50).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Information

Affiliations

Department of Microbiology and Molecular Genetics, Michigan State University, 567 Wilson Rd., Rm 2215, Biomedical and Physical Sciences building; East Lansing, MI 48824, USA.

Contributions

GR conceived the study and planned the sample collection protocol with contributions from KK. The three authors participated in the collection of the samples, J-YL processed them to extract DNA for sequencing and data analyses. GR and J-YL interpreted the results and wrote the manuscript with contributions from KK. All authors read and approved the final version of the manuscript.

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Figure Legends

Figure 1 Anatomy of the ear, pharynx and oral cavity.

(A) Anatomic structures in the outer, middle, and inner ear (illustration modified from Iain at the English Wikipedia, CC BY-SA 3.0). (B) Lateral cross section of the head showing the oral and nasal cavities, the three pharyngeal regions (nasal-, oral-, and laryngo-) and the mucosal folds around the ET orifice and torus tubarius (illustration modified from Sémhur at Wikimedia Commons, CC BY-SA 3.0). (C) Frontal view of the oral cavity (licensed from Biorender and edited to add labels).

Figure 2 Otic community diversity.

(A) Alpha diversity of the otic (blue), oropharyngeal (gray) and buccal (orange) communities based on richness (observed species), diversity (Shannon index) and evenness (Simpson index). Box plots show 50% of the diversity values in boxes, 25th and 75th percentiles as whiskers, median (line across the boxes), average (cross), outliers (circles outside the boxes) and confidence value from *t*-test comparisons (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; exact confidence values in Supplementary Table 4, , Additional File 1). Estimation graphics at the bottom show the mean (circle) diversity difference (Δ) of oropharyngeal or buccal samples versus the otic mean diversity (dashed blue line), the complete Δ distribution of values (shaded curve) and the 95% confidence interval of Δ (vertical line). (B) Principal Coordinates Analysis (PCoA) of weighted UniFrac distance in non-divers and divers showing the spatial clustering of otic (blue) and buccal (orange) samples and overlap of these clusters with the central oropharyngeal samples (gray). Axes PC1 and PC2 show the proportion (%) of variance explained.

Figure 3 Phylum-level composition of the otic communities in reference to oropharyngeal and buccal samples.

(A) Inter-individual differences and mean relative abundance (%) of phyla at each collection site (B) Distribution of abundance values (*top*) and estimation plots (*bottom*) for each of the four

dominant phyla in non-divers (nD) and divers (D) at each collection site. Estimation plots show the mean difference (Δ , solid circle), complete Δ distribution (shaded curve) of divers and 95% confidence interval of Δ (vertical line) for the divers cohort in reference to the non-divers (dashed horizontal line). Statistical values are available in Table S4, Additional File 1.

Figure 4 Distribution and abundance of otic, oropharyngeal and buccal genera.

(A-B) Core membership of genera shared by at least half of non-divers (nD) and divers (D) at each collection site **(A)** and among all microbiomes **(B)**. **(C)** Neutral model fit of otic community assembly with the oropharyngeal or buccal communities as potential sources (R^2 , goodness of fit). Gray symbols represent neutrally distributed OTUs (within 95% confidence interval around the best-fit). Taxa above (green) or below (red) the confidence interval are more likely to be positively (over-represented) or negatively (under-represented) selected in the middle ear, respectively. **(D-E)** Heatmaps of individual **(D)** or average **(E)** Z-score transformed relative abundance (normalized z-score > -1.0) of the 20 dominant genera (color-coded by phylum: Bacteroidetes, blue; Fusobacteria, yellow; Firmicutes, gray; Proteobacteria, orange; Actinobacteria, green; Spirochaeta, dark blue; SR1, light blue; Planctomycetes, dark gold).

Figure 5 Predicted otic microbiome functions.

(A) Metabolic structure of the otic, oropharyngeal or buccal communities of non-divers (nD; horizontal line) compared to divers (D). The lines connect the mean relative proportions of the 41 predicted functions and the most prevalent 22. **(B)** Dominant metabolic functions and pairwise comparisons of their abundance in the buccal (B) versus otic (O) or oropharyngeal (C; for center of the oropharynx) samples. Data from the statistical analyses is available in Table S4, Additional File 1.

Additional files

Additional File 1 - Supplementary Tables 1-4 and Supplementary Figures 1-6

- Supplementary Table S1: Age and gender (female, F; male, M) of study's participants.
- Supplementary Table S2: Diving experience (mean and standard deviation [SD] of total number of dives and diving depth in ft) reported by the participants in the diver's group ($n=9$).
- Supplementary Table S3: Mean relative abundance (% from Figure 3) and standard deviation (in parenthesis) of the 4 most abundant phyla in all the three samples collected in non-divers and divers.
- Supplementary Table 4: Summary of statistic values estimated from the data analyses in various figures.
- Supplementary Figure S1: Abundance patterns and distribution of dominant otic genera.
- Supplementary Figure 2: Gender distribution of most abundant genera in the otic and buccal communities of non-divers and divers.
- Supplementary Figure 3: Distribution of the most abundant genera in each collection site.
- Supplementary Figure 4: Oropharyngeal and/or buccal OTUs over-represented in the otic neutral model.
- Supplementary Figure 5: Correlation between the relative abundances (%) of OTUs in otic and potential source (oropharyngeal and buccal) communities.
- Supplementary Figure 6: Core metabolic structure of the otic communities and neighboring oropharynx and buccal mucosae.

Additional File 2 – Otic, oropharyngeal and buccal core microbiomes and shared membership.

Excel file with taxa in the core and shared microbiomes shown in Figure 4 (A-B).

Additional File 3 – Neutral model of otic community assembly

900 Excel file listing taxa predicted to be neutrally distributed, over-represented or under-represented
901 in the otic microbiome using the oropharyngeal and/or buccal communities as sources of microbial
902 immigrations.

Figures

Figure 1

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Supplementary Files

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