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Recent advances in understanding the natural history of the otitis media microbiome and its response to environmental pressures

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Abstract

Objective—To perform a comprehensive review of otitis media microbiome literature published between 1st July 2015 and 30th June 2019.

Data sources—PubMed database, National Library of Medicine.

Review methods—Key topics were assigned to each panel member for detailed review. Draft reviews were collated and circulated for discussion when the panel met at the 20th International

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Author Contributions

Each author compiled literature and drafted a section(s) of the manuscript. All authors contributed to drafting of the final manuscript.

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Symposium on Recent Advances in Otitis Media in June 2019. The final draft was prepared with input from all panel members.

Conclusions—Much has been learned about the different types of bacteria (including commensals) present in the upper respiratory microbiome, but little is known about the virome and mycobiome. A small number of studies have investigated the middle ear microbiome; however, current data are often limited by small sample sizes and methodological heterogeneity between studies. Furthermore, limited reporting of sample collection methods mean that it is often difficult to determine whether bacteria detected in middle ear fluid specimens originated from the middle ear or the external auditory canal. Recent *in vitro* studies suggest that bacterial interactions in the nasal/nasopharyngeal microbiome may affect otitis media pathogenesis by modifying otopathogen behaviours. Impacts of environmental pressures (e.g. smoke, nutrition) and clinical interventions (e.g. vaccination, antibiotics) on the upper respiratory and middle ear microbiomes remain poorly understood as there are few data.

Implications for practice—Advances in understanding bacterial dynamics in the upper airway microbiome are driving development of microbiota-modifying therapies to prevent or treat disease (e.g. probiotics). Further advances in otitis media microbiomics will likely require technological improvements that overcome the current limitations of OMICs technologies when applied to low volume and low biomass specimens that potentially contain high numbers of host cells. Improved laboratory models are needed to elucidate mechanistic interactions among the upper respiratory and middle ear microbiomes. Minimum reporting standards are critically needed to improve inter-study comparisons and enable future meta-analyses.

Keywords

Microbiota; otitis media; middle ear; nasopharynx; oropharynx; adenoids

Introduction

The otitis media (OM) microbiome refers to the full complement of microbes (bacteria, viruses and fungi) present in the upper respiratory tract (URT) and/or middle ear that can either directly or indirectly contribute to disease. A suite of methods has emerged in recent years that enable previously unimagined analysis of the often complex mucosal microbial communities associated with OM (Box 1). This review examines recent OM microbiome research with a focus on the natural history of the URT and middle ear microbiomes; environmental and clinical factors that may impact the OM microbiome; advances in understanding microbial interactions in the OM microbiome; and new methods for studying and modifying the OM microbiome. The aim of this review is to summarise recent advances and propose future research goals.

Methods

The PubMed database was searched for articles published between 1st July 2015 and 30th June 2019 using the MeSH terms otitis media and microbiota. Additional searches were performed using the following terms to capture relevant literature that may have been published with other keywords: otitis media, middle ear, nasal, or adenoid combined with

microbiome, 16S, metagenome, transcriptome, metabolome, virome, phage, fungus or sequencing; low biomass combined with microbiome, metagenome, transcriptome, metabolome or nested PCR; and microbiome and human DNA depletion. Articles not relevant to OM or the URT microbiome were excluded, as were articles with a primary focus on human genetics, otitis externa or the URT microbiome in patients with acute or chronic respiratory illness (e.g. pneumonia, cystic fibrosis, asthma). Abbreviations used in this review are summarised in Box 2.

Discussion

INTRODUCTION TO KEY CONCEPTS IN OM MICROBIOME STUDIES

Most OM microbiome studies have examined bacterial communities (referred to as the microbiota) in the URT and middle ear using 16S rRNA gene sequencing methods. Microbiota data require specialist analytic approaches that incorporate ecological concepts, including relative abundance and diversity. Relative abundance describes the proportion of a bacterial community occupied by each taxon and is used to assess dominance of individual taxa within polymicrobial communities. Diversity is described using alpha and beta diversity metrics. Alpha diversity is a within-specimen measure of microbial richness (number of different taxa) and/or evenness (proportional distribution of taxa) [1]. Alpha diversity metrics used in OM microbiota studies have included the Shannon's [2–5], Simpson's [6,7], Chao1 [2,5] and Faith's phylogenetic [2,6] indices. It is important to note that while these metrics describe the bacterial community structure, alpha diversity is not influenced by the types of taxa in the community. Thus, communities that contain vastly different bacteria can have similar alpha diversity if the overall bacterial community structure is similar. For example, there would be little difference in alpha diversity among bacterial communities containing 90% *Streptococcus pneumoniae* sequences and those containing 90% *Haemophilus influenzae* sequences. Alpha diversity measures may also be affected by the compositional nature of microbiota data, which may mask bacterial load effects [8]. For example, *H. influenzae* may be interpreted as having a greater role in infection when present at 90% versus 30% relative abundance; however, this may not necessarily be the case if the total bacterial loads are respectively 10^3 cells/ml (900 *H. influenzae* cells) versus 10^6 cells/ml (3×10^5 *H. influenzae* cells). Alpha diversity can also be affected by amplification biases that distort the relative abundance of different taxa [1]. Thus, care must be taken when comparing alpha diversity and the relative abundance of specific taxa between studies [1].

Beta diversity is a between-specimen measure of microbial community similarity. Common measures of beta diversity in OM microbiota studies include the Bray-Curtis [2,4,7,9] and UniFrac [2–4,6,9] metrics that are used to create a distance matrix describing the relatedness of different specimens. A range of tools can be used to visualise beta diversity [10], with principal coordinate analysis, non-metric multidimensional scaling and cluster analyses common among OM studies [3,6,7,9]. Formal testing of microbiomic differences between patient groups is commonly performed using the PERMANOVA test (also called the adonis test) [2,3,5,9,11,12].

IMPORTANT LIMITS IN THE RECENT OM MICROBIOME LITERATURE

Methodological heterogeneity emerged as a theme of this review. There is currently limited scope for comparing data across studies because of multiple sources of methodological variation, including: the OM diagnostic criteria; URT and middle ear sampling procedures; DNA extraction methods; inclusion of bacterial load measures; processing, analysing and reporting of laboratory controls; sequencing parameters; and analytic pipelines. The potential for methodological variation to bias microbiome data must be considered when comparing data from different studies [13–16] and must be addressed if OM microbiome data are to be useful to future meta-analyses.

Limitations of microbiota profiling performed with 16S rRNA gene sequencing

—Inherent amplification biases mean that a bacterium's relative abundance may vary depending on the region of the 16S rRNA gene that is analysed [16]. Thus, care must be taken when comparing data from studies that targeted different regions of the 16S rRNA gene. To date, the V1–3 [7,17], V3–4 [4,9,18], V4 [2,11,19,20] and V5–6 [21] regions have all been used in OM studies. A further limitation is that short-read 16S rRNA gene sequences may only provide sufficient taxonomic resolution for bacterial identification to the family or genus-level. This limitation can be especially problematic in OM studies where differentiation of recognised otopathogens from closely-related commensal species is needed (e.g. distinguishing *S. pneumoniae* from other Mitis Group Streptococci). In the future, this limitation may be overcome by using technologies that support full-length 16S rRNA gene sequencing, as recently demonstrated for sinonasal specimens from adults [22].

Challenges in characterising the microbiome when few bacteria are present—

Sequences arising from residual microbial DNA present in sterile laboratory reagents and kits can distort microbiota data when fewer than 1000 bacterial genomes are analysed [23–25]. Failure to address this issue can lead to false conclusions, as recently reported in placental microbiome studies [26]. Despite the risk, bacterial load is often not reported in OM microbiome studies [2,4,5,9,17,19,20]. Among studies that have considered bacterial load limitations, there is heterogeneity in the quantification methods; inclusion, sequencing and reporting of negative controls; and the analytic methods used to identify potential contaminants [7,9,11,18,21]. A standardised approach to bacterial load issues is needed to assist inter-study comparisons of OM microbiome data.

RECENT STUDIES CHARACTERISING THE OM MICROBIOME

Regardless of the limitations described above, OM microbiome studies have revealed much new knowledge about the types of bacteria present in URT and middle ear specimens. Recent advances challenge our understanding of microbial dynamics that may be important in OM pathogenesis and are driving development of new therapies. Here we review recent findings about the natural history of the OM microbiome.

Advances in understanding the natural history of the URT microbiome—The URT harbors a complex ecology of bacteria, viruses and fungi that can influence each other and impact the host's health and disease [27–29]. The URT includes the nares, nasal passages, sinuses, nasopharynx, adenoid pad and oropharynx [29]. These anatomic sites

have distinct physiological and microenvironmental differences [28,30] that create discrete ecological niches. The adenoid and the nasopharynx are generally considered the URT sites most relevant to OM [3,27].

Recent OM microbiome studies that included nasal or nasopharyngeal (NP) specimens have focused on pediatric populations [3,4,6,7,18,19,31], reflecting the higher OM disease burden among those aged <5 years [32]. A similar trend towards pediatric populations was observed for studies of the NP microbiome in healthy individuals and in other respiratory disease contexts [21,29,33–45].

The composition and relative abundance of taxa among the NP microbiota change with age. Stearns *et al.* [33] showed that while the NP microbiota between parents and their young children was somewhat similar, *Staphylococcus epidermidis*, *H. influenzae*, *H. parainfluenzae*, *Dolosigranulum pigrum*, *Corynebacterium durum*, and *C. mucifaciens* were present at higher relative abundances in children. Prospective longitudinal birth cohort studies in Europe [35,36,46] and Asia [34] demonstrated distinct patterns of NP microbiota succession as infants age. In a healthy Dutch infant population, an initial dominance of *Streptococcus* was rapidly replaced by *S. aureus* within the first week of life, followed by *Corynebacterium* spp. and *D. pigrum* and subsequently *Moraxella* spp. after 2–3 months of age [35,36]. Similar NP bacterial populations were described for healthy children from Australia and China [6,37], suggesting that a common NP microbiota composed of a few highly abundant species is associated with health in young children. Age-dependent NP microbiota profiles were also reported by a study of 286 children (aged <7 years) with acute otitis media (AOM); dominant taxa in this cohort were *Moraxellaceae*, *Staphylococcaceae*, *Corynebacteriaceae*, *Streptococcaceae* and *Pasteurellaceae* (the family which includes *Haemophilus*).

Several studies found that the composition and order of succession of the NP microbiota in infants was related to subsequent respiratory disease risk [28,38,46,47], with decreased NP microbiota stability [35,39] and early dominance by *Moraxella* [35,46] associated with an increased risk of acute respiratory tract infection (ARI). Prolonged reduction in the abundance of *Corynebacterium* spp. and *D. pigrum* during the first year of life was also associated with an increased ARI rate in Dutch children [35], suggesting potentially protective effects from these taxa. Similarly, a recent Italian study reported higher relative abundance of *Corynebacterium* spp. and *D. pigrum* in healthy children compared with those with recurrent AOM; this difference was even more pronounced when compared with children with recurrent perforations of the tympanic membrane [48]. *D. pigrum* was not a dominant taxon among infants living in the Maela refugee camp on the Thai-Myanmar border (only detected at <3% relative abundance), whereas the prevalence and abundance of the newly described *Candidatus* *Ornithobacterium hominis* increased with age [34,49]. Lower diversity was observed among the nasal microbiota of 73 New Zealand children with chronic OME (associated with higher relative abundance of *Streptococcus*, *Moraxella* and *Haemophilus*) compared to 105 controls [50].

Acute respiratory viral infections may alter the NP microbiota [19,39–45], with some studies reporting an association with increased *Haemophilus* relative abundance [41–44] along with

Streptococcus, *Achromobacter*, *Prevotella* and *Veilonella*. Asymptomatic viral infections are common [51]; however, the significance of this to microbiome dynamics and OM pathogenesis is not known. A limitation of current literature is that only selected human pathogenic viruses were tested in most OM-relevant studies [51–57]. The sole metagenomic study describing the nasal microbiome found viral diversity was independent of geographic location and that novel viruses were present, including viral families not previously thought to infect humans [58]. No OM-relevant studies investigated the NP mycobiome (a measure of fungal communities).

Although URT sampling is commonly used as a surrogate measure of middle ear microbiology, multiple studies found the URT and middle ear microbiota were distinct [3,4,6,7,18,21,52,56], consistent with niche-specific environmental selective pressures. The adenoid pad contains a more diverse microbiota than the nasopharynx [7]. Recent studies support the hypothesis that the adenoid pad is a reservoir of microbes that can cause ARI [31,59], but there have been conflicting reports for OM [3,4,18,60]. Despite the overall differences between the microbiota of the middle ear and URT, several studies reported co-detection of *Haemophilus*, *Streptococcus* and *Moraxella* across anatomical sites [5–7,21]. Boers *et al.* [21] observed that *Haemophilus* and *Streptococcus* were present in middle ear fluid (MEF) only when also present in the nasopharynx. *Alloiococcus* and *Turicella* were commonly found in the MEF, but not in the nasopharynx [6,21], whereas *Gemella* and *Neisseria* were highly abundant in the nasopharynx of OM patients [6,21], but not in healthy controls [6].

Advances in understanding the natural history of the middle ear microbiome

—Recent studies have revealed new knowledge about the composition of the middle ear bacterial communities; however, generalisability of findings across populations and diagnostic groups remains challenging as there are few data. The search terms used identified 15 middle ear microbiome studies since June 2015 that included a combined total of 722 subjects. The sample size included in middle ear microbiome studies ranged from 4–155 subjects (Supplementary Table). This relative scarcity of data may relate to the technical challenges inherent to analysing low volume and low biomass middle ear specimens [61]. Multiple studies reported excluding samples because insufficient bacterial DNA was recovered or too few sequences were generated (13–65% of middle ear specimens were excluded from some studies [5–7,9,21,61]). Other studies overcame bacterial load limitations by including a nested PCR when preparing the amplicon library [9,61].

The clinical significance of variable bacterial load in middle ear specimens is not yet clear. In healthy individuals, the middle ear is expected to have few (if any) resident bacteria due to the host defences that have evolved to protect against infection. Consistent with this, Neeff *et al.* [9] cultured no bacteria from surgically collected middle ear swabs from 22 control patients (healthy middle ears); 68% were also negative for bacteria using molecular methods. In contrast, Minami *et al.* [11] were able to characterise the microbiota in middle ear swabs from 66/67 control patients and reported significant differences among children (aged <14 years) and adults (aged >14 years; PERMANOVA $p=0.002$); Proteobacteria was the most dominant phylum in children whereas the phylum Firmicutes was more dominant in adults. It is unclear whether these contrasting findings reflect methodological variation

between the two studies or natural variation in the middle ear microbiome that may be missed when studying small cohorts. Improved understanding of the healthy middle ear microbiome is needed to inform the design of future studies that aim to understand the initiation of an OM episode. For example, it is not yet clear whether OM may be initiated by dysbiosis of a resident middle ear microbiome or is solely due to an acute infection driven by an invading pathogen (or a combination of these mechanisms).

Studies of patients with otitis media with effusion (OME), AOM or recurrent AOM (rAOM) found operational taxonomic units (OTUs) consistent with classical otopathogens among the middle ear microbiota (Table 1), with *Haemophilus*, *Streptococcus* (including the Mitis group and Group A *Streptococcus*) and *Moraxella* commonly reported as the dominant taxa in middle ear specimens [5,7,61,62]. Other bacteria common to patients with OME and AOM include *Alloiococcus*, *Corynebacterium*, *Pseudomonas*, *Staphylococcus* and *Turicella* spp. (Table 1); however, the significance of these taxa is unclear as all may be normal flora of the external auditory canal (EAC) [6] and, thus, may indicate contamination of the middle ear specimen during sampling. Conversely, detection of EAC flora may indicate secondary middle ear infection following tympanic membrane perforation or placement of tympanostomy tubes. Indeed, otopathogens, EAC flora and a range of other taxa are among the bacteria identified in middle ear specimens from patients with chronic perforations and those with tympanostomy tubes *in situ* (Table 1), including middle ear swabs from patients with chronic suppurative OM (CSOM) that were collected intraoperatively [9]. Improved methods for assessing the likelihood of EAC contamination during sampling are needed to aid interpretation of middle ear microbiome data. Recent efforts to address this issue include comparison of paired EAC and middle ear specimens [4,6]. For example, Lappan *et al.* [6] found similar taxa among paired EAC and middle ear specimens from children with AOM; however, the relative abundance of some taxa varied depending on the specimen type (*Alloiococcus* and *Turicella* were detected at higher abundance in EAC specimens whereas *Haemophilus* was more dominant in middle ear specimens). Further work is needed to define best practice for preventing contact with the EAC during middle ear sampling.

Middle ear microbiome data derived from other OMICs technologies are largely lacking. The sole study [63] that used metagenomics to analyse middle ear specimens from OM patients found the sequence data were dominated by human reads (91.3% $\pm$ SD2.9%); microbial data from this analysis were not reported. Proteomic characterisation of MEF was more successful and revealed a relationship between the MUC5B mucin and neutrophil extracellular traps [63]. Studies of the middle ear virome and mycobiome are currently lacking. One study examined the mycobiome of the EAC among 17 healthy adults and found lower fungal diversity than other body sites with a total of only 10 taxa detected (Table 1). *Malassezia* species were present at >90% relative abundance in 14/17 subjects, suggesting an important role for this genus in this environment [64].

Studies relating middle ear microbiota to host responses are beginning to emerge. Krueger *et al.* [2] reported significant differences in middle ear microbiota when grouping 55 OME patients by age, hearing loss and mucin type (PERMANOVA $p=0.029$, 0.019 and 0.013 , respectively); *Turicella* and *Pseudomonas* were more abundant in children with OME aged <24 months, whereas *Haemophilus* was associated with hearing loss and co-detection of

MUC5AC and MUC5B mucins. Studies investigating relationships between the gut microbiome and middle ear disease have been proposed [65], but data are not yet available.

ENVIRONMENTAL IMPACTS ON THE OM MICROBIOME

A range of environmental factors may affect the composition and behaviour of the URT and middle ear microbiomes, extending from clinical interventions such as vaccines and antibiotics through to diet and environmental variables (e.g. smoke exposure) [28]. Here we review current understanding of relationships between these environmental factors and the OM microbiome.

Vaccine impacts on the OM microbiome—Paediatric pneumococcal conjugate vaccination (PCV) programs have altered the pneumococcal population, eradicating or significantly reducing carriage and disease caused by vaccine serotypes [27,66]. However, in the developing world, some PCV-types persist despite vaccination and most regions with a PCV program have experienced replacement of vaccine-serotypes with non-vaccine serotypes in carriage, OM, pneumonia and invasive pneumococcal disease [67]. It is less clear whether PCV and other respiratory vaccines have impacted the broader respiratory microbiome, as only four studies of PCV impacts on the URT microbiome were identified, all in paediatric populations, and all of which were limited by small sample sizes.

The first study by Mika *et al.* [68] compared aggregated longitudinal NP microbiota data (fortnightly swabs) in healthy Swiss infants (aged < 1 year) who received two or more doses of either PCV7 (n=20) or PCV13 (n=21). Children vaccinated with PCV13 had less pneumococcal carriage (Linear mixed effect model; LME $p=0.01$), higher Shannon's diversity index ($p=0.01$), richness (including significantly more *H. influenzae* oligotypes) and more stable microbiota (Jensen-Shannon distance LME and t-test; $p=0.03$) compared with PCV7-vaccinated children.

The second study [69] was a randomised controlled trial in Kenya that compared the NP microbiota of children (aged 1 to 5 years) given two doses of PHiD-CV10 (n=30) or Hepatitis A vaccine (n=30) two months apart. The median age of children in both groups at the time of vaccination was 31 months. At 6-month follow-up, there was no difference in the composition or abundance of otopathogens, including prevalence of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* or *S. aureus*; however, children in the control group experienced a larger but non-significant increase in the prevalence of *S. pneumoniae* over the duration of the study (14% versus 4% increase, $p = 0.36$) compared to those in the PHiD-CV10 vaccinated group. On an individual level, the microbiome composition shifted between baseline and follow-up, independent of age, sex, school attendance, antibiotic exposure, and vaccination, highlighting the natural temporal fluidity of the NP microbiota among children in this age group. In contrast to the study by Mika *et al.* [68], non-pneumococcal replacement appeared to be limited in the Kenyan setting. In longitudinal comparison (baseline versus 6 months), only the Hepatitis A-vaccinated children experienced a decrease in alpha diversity.

The third study [70] compared the longitudinal (sampled bi-weekly for 6 months then bi-monthly for 6 months) NP microbiota of two groups of PCV7 vaccinated Gambian infants

(PCV naïve, n=30; PCV non-naïve, n=39) with an unvaccinated control group (vaccine naïve, n=33) to determine the impact of a three-dose schedule. They found no significant differences in the Shannon's diversity index or richness of the NP microbiota in the vaccinated compared to non-vaccinated infants in the first year of life, but did see changes in the relative abundance of *Streptococcus*, *Moraxella*, *Dolosigranulum*, *Haemophilus*, *Pseudomonas* and *Pelomonas*. Importantly, clearance of pneumococcal vaccine serotypes and concurrent replacement with non-vaccine serotypes occurred within two-weeks of the first PCV7 dose among vaccinees.

The fourth study [71] found no difference in NP carriage of *S. pneumoniae*, *S. aureus*, *H. influenzae* or *M. catarrhalis* among PHiD-CV10 versus unvaccinated Brazilian children aged 6–23 months, based on 16S rRNA gene sequencing analysis and pathogen transcriptomes. The *S. pneumoniae* metabolic rate (species-specific RNA:DNA ratios) was higher in controls compared to vaccinated children, suggesting a pathogen-specific effect that is more subtle than that represented by carriage alone [71].

Other vaccines may also affect the NP microbiota [72,73]. For example, among 17 healthy adult volunteers, intranasal inoculation with live attenuated influenza vaccine (n=10) was associated with significant changes in the NP microbiome after 1–2 and 4–6-weeks follow-up, compared with no changes observed over the same time period among controls (n=7) who were inoculated with a nasal saline spray [72]. Other emerging evidence suggests that the respiratory and gut microbiome may influence host-mediated vaccine immune responses (reviewed by Ciabatti *et al.* [74]), and thereby affect the response of the URT microbiome to vaccination. Relationships between such host-mediated effects and OM remain to be determined.

Collectively, these data suggest that the direct impact of PCVs on pneumococcal populations also has an indirect impact on the wider respiratory microbiota. Larger studies are needed to confirm patterns of vaccine impact on the NP microbiota and to determine the implications of such impacts on OM risk.

Impacts of antibiotics on the OM microbiome—Antibiotic treatment of OM can result in the development of antibiotic resistance, not only among the otopathogens for which antibiotics were prescribed, but also among the wider microbiota which, in turn, can serve as a reservoir of resistance genes [75]. For example, antibiotic stress induces transformation in naturally competent pneumococci, which can acquire resistance genes from related *Streptococcus* species in the respiratory microbiome [76]. The acquisition of resistance is further promoted by persisting sub-inhibitory concentrations of antibiotic post-treatment that increase the mutation frequency in pneumococci. The number of courses of antibiotics, as well as the dosage and duration of treatment can contribute to antibiotic resistance and may influence changes in the NP microbiome [77]. Indeed, antibiotic induced changes in the microbiome may be cumulative in an individual where species with important metabolic functions are lost [78]. Resistance to antibiotics prescribed in primary care can persist for up to 4 years [79].

Antibiotic treatment can also disrupt the ecology of the microbiome [75], resulting in dysbiosis [80–82]. A dysbiotic respiratory microbiome facilitates acquisition of new bacterial or viral pathogens, carriage of multiply resistant bacteria, or viral co-infections [82]. An early study of the NP microbiota of infants with AOM [83] found that recent antibiotic treatment was the strongest factor for inducing changes in the NP microbiota, with a reduced prevalence of *Streptococcaceae* (including pneumococci) and *Corynebacteriaceae*, increased prevalence of *Enterobacteriaceae* and greater relative abundance of *Pasteurellaceae* (the family that includes *Haemophilus*). More recent longitudinal studies of American [19] and Dutch [35] children during the first year of life also found antibiotic treatment reduced the relative abundance of *Corynebacterium* spp. and *D. pigrum*. Antibiotic use was associated with an increase in alpha diversity among the NP microbiota of American infants [19], whereas this relationship was not evident among longitudinally-followed infants residing in the Maela refugee camp [34].

Other studies have demonstrated the wider potential impacts of antibiotics. For example, maturation of the immune system may be influenced by the gut microbiota composition over time [84]. Antibiotic perturbation of the gut microbiome could thus affect immune maturation with potential flow-on effects due to immune-mediated selective pressures on the microbiomes at other mucosal sites, including the URT. More detailed study is required to determine whether antibiotic-mediated perturbation of the composition and diversity of the gut microbiome may affect OM risk.

Impact of vitamin D supplementation on the OM microbiome—Low vitamin D levels in pregnancy and infancy are consistently demonstrated as a risk factor for subsequent ARI in children [85]. The mechanisms by which vitamin D might protect against ARI include enhanced anti-microbial peptide expression, regulation of inflammatory responses or support of commensal bacterial growth [86]. Two studies examined relationships between circulating vitamin D levels and the composition of the URT microbiota, both in patients with respiratory disease. Toivonen *et al.* [46] showed that the NP microbiota of infants hospitalised with bronchiolitis (n=1005) had a reduced richness ($p<0.05$) and Shannon Index ($p<0.05$) where circulating 25 hydroxy vitamin D (25OHD) levels were <66 nmol/L compared to 66 nmol/L. While the relative abundance of the 10 most abundant genera was similar by 25OHD status, the overall composition and structure of the microbiota profiles differed significantly ($p=0.04$). *Haemophilus*-dominant microbiota profiles were associated with significantly higher risk of intensive care admission (OR: 3.08, 95%CI: 1.31–7.25, $P=0.01$) among patients with lower vitamin D levels. The second study examined sputum and gut microbiota in a small vitamin D intervention trial (50,000 IU of vitamin D per week for 12 weeks vs placebo) in 41 adults with cystic fibrosis. In those with 25OHD <75 nmol/L at baseline, Gammaproteobacteria were detected at higher relative abundance in the gut, while Bacteroides were more abundant in the airway. At follow-up, *Lactococcus* was enriched in the gut of participants in the vitamin D group, whereas *Veillonella* and *Erysipelotrichaceae* were enriched in the placebo group. Changes in the airway microbiota were less well described, though *S. aureus* (implicated in pulmonary infections in cystic fibrosis patients) was highlighted as significantly enriched in the airway of those in the placebo group only. No studies have examined vitamin D in relation to the OM microbiome; however, a

randomised controlled trial showed that vitamin D supplementation (1000 IU/day) of otitis-prone Italian children (age 1–5 years) reduced the risk of AOM compared to placebo (44% vs 66%; $p=0.03$) [87]. Further research is needed to determine whether vitamin D acts as a mediator of the OM microbiome.

Impact of breast feeding on the OM microbiome—Breast feeding is demonstrated to have a protective effect against respiratory infections during the first 6 months of life [88], attributed to secretory IgA, oligosaccharides, antimicrobial and immune stimulating factors [89]. Furthermore, breast milk has its own microbiota which, while highly variable between individuals, is dominated by Proteobacteria and Firmicutes (*Streptococcus* and *Staphylococcus* are the most abundant genera) [90]. No recent work relating breast feeding to the OM microbiome was identified; however, two earlier studies examined associations between breast feeding and the NP microbiota. Biesbroek *et al.* [91] prospectively observed 101 infants that were exclusively breastfed and 101 were exclusively formula fed from birth. At 6-weeks of age, breastfed infants had an increased presence and abundance of *D. pigrum* (relative effect size (RES), 2.61; $p=0.005$) and *Corynebacterium* spp. (RES, 1.98; $p=0.039$) and decreased abundance of *Staphylococcus* (RES, 0.48; $p=0.030$) and anaerobic bacteria, such as *Prevotella* (RES, 0.25; $p<0.001$) and *Veillonella* (RES, 0.33; $p=0.001$). *D. pigrum* and *Corynebacterium* spp. were dominant (>50% of profile) in 45% of breast fed infants and only 19% of formula fed infants (relative risk, 2.37; $P = 0.006$). Furthermore, the presence of *D. pigrum* was inversely associated with wheezing and respiratory tract infections. By 6-months of age these associations were no longer evident. In a second prospective study of 112 infants, Bosch *et al.* [35] used a machine learning algorithm to demonstrate that children experiencing a higher number of ARIs ($n>2$) in their first year of life had aberrant NP microbiota development from their first month compared to infants who had few ARIs ($n = 2$). This included extended reduction in *Corynebacterium* spp. and *D. pigrum* and later enrichment of *Neisseria* and *Prevotella* spp. Breast feeding and vaginal birth were associated with prolonged dominance of the NP microbiota by *Corynebacterium* spp. and *D. pigrum*, and late enrichment of *Moraxella* spp. Conversely, formula-fed and/or caesarean-born children had a higher abundance of *Gemella*, *Streptococcus*, *Neisseria* spp. and (facultative) anaerobes, including *Prevotella*, *Granulicatella*, and *Actinomyces* spp. after the first month of life.

Impact of tobacco and environmental smoke on the OM microbiome—Noxious particles and gases in tobacco and environmental smoke cause inflammation of the respiratory mucosa that increases mucus production and down-regulates mucosal IgA, production of antibacterial molecules and phagocytic activity [92]. For children, it is well established that exposure to environmental tobacco smoke increases the risk of OM [93]; however, no studies investigating the effect of smoke exposure on the URT microbiome of children were identified. Among adult smokers (current or previous, $n=44$) the NP microbiota had lower richness and evenness compared to adults who had never smoked ($n=57$) [94]. Notably, *Staphylococcus* species had a higher relative abundance among smokers (52.4% vs 19.4% median relative abundance; $p = 0.005$) [94]. In contrast, among 1204 American adults [95], there was no difference in the microbiota of buccal cells between former and never smokers [95]. Other studies showed little to no difference

between the URT microbiota between smokers and non-smokers [96–98], whereas differences in viromic and metabolic profiles were identified in the lung [97]. Phages were the most common viruses detected; *Prevotella* phages were >2-fold higher in smokers whereas *Lactobacillus* and *Gardnerella* phages were 10-fold more abundant in non-smokers. Overall, smokers exhibited fewer viral populations and a less diverse viral ecology [97].

RECENT ADVANCES IN UNDERSTANDING POLYMICROBIAL INTERACTIONS IN OM

Existing data hint at a complex network of interspecies interactions that shape the composition of bacterial microbiota from the nostrils through to the nasopharynx, potentially impacting on OM risk. However, only a few of these interactions are defined, e.g. effects of influenza virus infection on *S. pneumoniae* colonisation and virulence [99]. Others await investigation, such as the possible influence of bacterial microbiota on host response to viral infection [100]. Here, we review recent bacterial-bacterial and bacterial-host interactions that identify ways by which commensal bacteria might limit colonisation by, or virulence of, otopathogens. The goal of such research is to identify native URT beneficial bacteria that are candidates for use as probiotics to prevent acute or recurrent OM via colonisation resistance (bacterial interference). Although beyond the scope of this review, there is literature related to cystic fibrosis and chronic wounds on interactions between commensals and *P. aeruginosa* that might provide insights for chronic OM. Additionally, it is important to note that new research continues to characterize the effects of microbial interactions on the multispecies bacterial biofilms implicated in middle ear infections, e.g. *S. pneumoniae* and nontypeable *H. influenzae* (NTHi) [101,102]; NTHi and *M. catarrhalis* [103,104]; and NTHi and *Alloiococcus otitidis* [105], although a role for the latter in OM is unclear.

Associations in compositional studies of URT microbiota have stimulated exploration of molecular mechanisms by which commensal members of the genus *Corynebacterium* (phylum Actinobacteria) interact directly or indirectly with *S. pneumoniae* and with *S. aureus* (both phylum Firmicutes). Four *Corynebacterium* species are commonly present in the human nasal passages/nasopharynx: *C. accolens*, *C. tuberculostrictum*, *C. pseudodiphtheriticum* and *C. propinquum* [106]. Among these, *C. accolens* excretes a triacylglycerol lipase, LipS1, that hydrolyzes model host surface triacylglycerols releasing free fatty acids that inhibit *S. pneumoniae* *in vitro* [107]. Thus, high-levels of *C. accolens* might modify the nasal habitat to be inhospitable for *S. pneumoniae*. *C. propinquum*, on the other hand, sequesters iron from coagulase-negative *Staphylococcus* species (CoNS) *in vitro* by excreting the siderophore dehydroxynocardamine. This siderophore biosynthetic gene cluster is transcribed *in vivo* in human nostrils [108], a reminder that metal-competition in nasal microbiota deserves further investigation. *C. pseudodiphtheriticum* exhibits contact-dependent killing of *S. aureus* via a yet to-be-identified mechanism requiring *S. aureus* production of phenol-soluble modulins, such that resistant *S. aureus* mutants display attenuated virulence [109]. Also, nasal priming with *C. pseudodiphtheriticum* strain 090104 alters host response to respiratory syncytial virus infection and secondary *S. pneumoniae* infection in a mouse model [110]. Finally, multiple species of *Corynebacterium* excrete an unidentified factor that inhibits *S. aureus* Agr quorum-sensing autoinducing peptides. This shifts *S. aureus* toward commensal behaviour *in vitro* and decreases *S. aureus* success in an abscess model during coinfection with *Corynebacterium* compared to mono-infection [111].

Other cross phylum interactions between the *Cutibacterium* (formerly *Propionibacterium*) *acnes* (Actinobacteria) and *Staphylococcus aureus* (Firmicutes) occur (reviewed by Brugger *et al.* [112]) but are likely to be less relevant in young children since *C. acnes* only becomes numerically dominant in the nose late in puberty [113].

Competition between other commensals, e.g. *C. propinquum* (above) and CoNS might have indirect effects on *S. aureus* colonisation via competition among *Staphylococcus* species. Recent insights into this long-recognised competition within the genus *Staphylococcus* include identification of additional CoNS-produced antibiotics that inhibit *S. aureus* growth, e.g., lugdunin by *S. lugdunensis* [114], lantibiotics by *S. hominis* [115], and CoNS-produced inhibitors of *S. aureus* Agr quorum sensing that attenuate *S. aureus* virulence, e.g. *S. caprae* AIP [116] and *S. hominis* AIP [117] with these two CoNS likely more important on skin. *Staphylococcus* species also display *in vitro* competition with non-staphylococcal nasal commensals [118,119]. Collectively, these studies point to a probable wealth of unrecognised interspecies interactions in the nasal passages that might impact the risk and clinical progression of OM.

Research is needed to elucidate the many molecular mechanisms by which commensal nasal/NP bacteria interact with otopathogens, and with each other, to identify compounds and strains that are candidates for future therapeutic use. In particular, there is a dearth of functional information about *D. pigrum* (Firmicutes) [120], which is positively associated with the genus *Corynebacterium* in many URT microbiome studies and negatively associated with *S. pneumoniae*, either alone or in conjunction with *Corynebacterium* (reviewed by Bomar *et al.* [121]). *D. pigrum* can be grown as *in vitro* biofilm [122]. Similarly, little is known about interactions of nasal commensals with either *H. influenzae* or *M. catarrhalis* or about potential effects of either phage or fungi on nasal bacterial microbiota and the risk of OM.

ADVANCES IN FUNCTIONAL OM MODEL SYSTEMS FOR STUDYING MICROBIAL INTERACTIONS

There are currently no experimental models that fully replicate the development or progression of chronic OM in humans (reviewed in Bhutta *et al.* [123]). The most relevant data on the host microbiome during otopathogen colonisation, and acute and chronic OM are those obtained from clinical specimens or from human challenge models, such as those that have been established for NTHi [124] and *S. pneumoniae* [125]. Cell culture and animal OM models do, however, remain useful tools for understanding microbial interactions, OM pathogenesis, and assessment of novel therapies. Recent advances include a murine middle ear epithelial cell model that was developed with an air-liquid interface to reproduce the native murine middle ear epithelium [126]. The primary middle ear epithelial cells in this model had similar morphology to the murine middle ear, with tight junctions formed between cells, presence of ciliated and non-ciliated cells and expression of epithelial cell markers. The major otopathogen NTHi was shown to interact with these cells, including intracellular invasion and induction of an inflammatory response [126].

While cell culture models enable host-pathogen interactions to be assessed at the epithelial surface, they do not fully recapitulate the complexity of OM, including consideration of the

host respiratory microbiome and its involvement in OM pathogenesis. Mice, rats and chinchillas remain the preferred animal models for OM research. While these animal models can provide insight into microbial interactions in the URT before, during and after experimental OM, there are limitations, most notably that the major human otopathogens are not natural colonisers or pathogens of rodents. It may be that rodent equivalents of the human otopathogens are best assessed in these animal models. For example, a recent AOM model used a natural otopathogen of mice, *Bordetella pseudohinzii* [127]. This model permits assessment of the natural development of AOM, and, in future, could be used to understand the role of the host microbiome in protection against OM. In addition, Kirkham *et al.* [128] recently demonstrated that a commensal of the murine respiratory tract, *Muribacter muris* (a Pasteurellaceae; the same family as *Haemophilus*) can be delivered intranasally to prevent NTHi colonisation and development of OM in mice. This work is based on the concept of microbial interference, where commensal bacteria compete with pathogens for binding sites and nutrients to beneficially alter the host microbiome. Bacterial interference strategies are currently being explored to combat many diseases (Table 2), including prevention of pneumococcal OM in children [129], and has the potential to significantly reduce antibiotic use. Current data suggest that for clinically-relevant microbial interference, the commensal must be from the same bacterial family as the target pathogen and occupy the same niche within the host.

TRANSLATION OF MICROBIOME DATA INTO CLINICAL THERAPEUTICS

Probiotics are defined by the World Health Organization as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [130]. Probiotics may have a role in preventing or disrupting infectious diseases of mucosal surfaces. In OM, there are three primary modes of probiotic administration: i) Direct application of probiotic species with a nasal spray or drop to access the NP space; ii) Direct application of probiotic eardrops through a perforated tympanic membrane (permitting direct access to the middle ear cavity); and iii) Oral probiotic intake, which could potentiate migration of probiotic species from the oral cavity to the nasopharynx, or have a systemic effect through gut-immune system interactions.

In earlier studies of nasal spray probiotic interventions in children with OM, Tano *et al.* [131] randomised 43 children to receive 4-months treatment with a daily nasal spray containing 5 α -haemolytic *Streptococcus* strains. No effect on AOM was recorded and no significant changes in NP colonisation by otopathogens was detected. Similarly, Skovbjerg *et al.* [132] compared a nasal spray probiotic treatment containing *S. sanguinis* and *Lactobacillus rhamnosus* versus placebo for 10 days before surgery for secretory OM in 60 children; the treatment did not alter the composition of the NP flora. More recent data are scarce; our search identified only one study published during the past 4 years. Marchisio *et al.* [129] used a single strain nasal spray of *S. salivarius* 24SMB in a randomised placebo controlled trial in 97 otitis-prone children. The spray was delivered twice daily for 5 days per month over 3 months. At the end of the trial, 50% of the children in the treatment arm were colonised with *S. salivarius* 24SMB and 28% remained colonised for a further 2 months. Fewer children experienced AOM in the treatment arm compared with placebo (30% vs 15%; $p = 0.076$). In addition, antibiotic use during the study period was lower in

children treated with *S. salivarius* 24 SMB than placebo (70 vs 83%; $p = 0.13$). This study suggests that intranasal administration of *S. salivarius* 24SMB may reduce the risk of AOM in otitis-prone children.

No studies have reported administration of probiotic eardrops directly into the ear space, and two studies (only one in past 4 years) reported the effect of oral probiotic supplements on NP microbiota on OM. In a large ($n=309$) double-blind, placebo-controlled study from Finland, otitis-prone children (10 months - 6 years) consumed either one probiotic capsule (*L. rhamnosus* GG and LC705, *Bifidobacterium breve* 99 and *Propionibacterium freudenreichii* JS) or placebo daily for 24-weeks. No differences were observed in colonisation prevalence of *S. pneumoniae* and *H. influenzae*, whereas the prevalence of *M. catarrhalis* increased in the probiotic group [133]. In contrast, a recent small observational study from Spain using oral treatment with *L. salivarius* PS7 in 64 otitis-prone children showed a reduction in AOM (historical controls) and a reduction of the number of positive swabs from the EAC; *A. otitidis* reduced from 12 to 2 positive participants, *H. influenzae* from 7 to 1, *M. catarrhalis* from 14 to 3, *P. aeruginosa* from 4 to 0, and *S. pneumoniae* from 9 to 3 [134]. These results are inconclusive, given the small numbers and comparison with an historical cohort, but support further investigation of this probiotic.

One study investigated the potential for *L. rhamnosus* GG (L GG) to colonise the middle ear after oral intake [135]. Forty children on the waitlist for myringotomy were randomised to either L GG or placebo for 3 weeks. Results from middle ear effusions showed that 4 of 20 patients in the L GG supplement group had L GG present compared with 1 of 20 patients in the placebo group. There was no difference in the abundance of *H. influenzae* or rhinoviruses in the middle ear, which may challenge the role of L GG as a disease modifier in OM. In contrast to the rather low rate of L GG detection in middle ear effusion, the same research group detected L GG in the adenoids of 100% of 20 children (aged 1–5 years) receiving L GG [136]; however, L GG was also detected from the adenoids of 76% of 20 children (aged 1–5 years) who received placebo and with no difference in the rhinovirus detection rate, further suggesting that the role of resident L GG in the airway is unclear. These results also demonstrate that the presence of a probiotic strain is not a reliable surrogate marker for clinical efficacy.

Another potential mechanism for impacting the NP microbiome is through administration of prebiotic supplements. Prebiotics are substrates that are “selectively utilized by host microorganisms conferring a health benefit”[137]. Although there are no recent studies, a 2013 study [138] compared oral probiotic supplement (*Streptococcus thermophilus* NCC 2496, *S. salivarius* DSM 13084, and *L. rhamnosus* LPR CGMCC 1.3724) with prebiotic supplement (Raftilose, a prebiotic fructooligosaccharide derived from inulin) versus placebo. There were no differences in AOM prevalence and no differences in NP prevalence of otopathogens between groups.

Thus, there are currently insufficient data to draw any firm conclusions about the effectiveness of probiotics or prebiotics for preventing or treating OM. Current data are limited by the variability in results and heterogeneity in strain selection, delivery method and treatment duration. Further studies are warranted and development of standardised reporting

should be high on the agenda in the OM scientific community. Reporting should ideally include both abundance and prevalence of pathogens in the URT (and MEF, where possible), as well as data on the commensal flora, including diversity and whether colonisation with the administered probiotic species occurred.

ADVANCES TOWARDS OM MICROBIOME META-ANALYSES

Due to the expanding use of electronic record keeping for health data, there has been an increase in the use of large, system-derived datasets and data linkage projects in health research [139]. In OM, studies using large data resources (e.g. hospital and government datasets) are emerging, including linkage of medical presentation and management data, such as emergency room and primary health care presentations for OM [140], records of grommet insertions after vaccination program implementation [141], and records of grommet insertions in health insurance databases [142]. In these settings, comprehensive conclusions can only be drawn where quality data exist and are managed by a centralised tool. One successful example is the Icelandic whole population study of the impact of the PHiD-CV10 vaccine on all cause AOM, which showed a reduction of 40% in children aged <4 months, and a 6 to 23% reduction in children aged 5 months to 3 years when comparing children born in the PHiD-CV10 era to children born prior to vaccine program implementation [143].

No studies to date have combined large data resources with OM microbiome datasets. Furthermore, heterogeneity among current OM microbiome studies may be a barrier to future data linkage studies. Current sources of heterogeneity include OM diagnostic criteria, clinical management, and application of molecular and microbiological testing methods. Opportunities to address this heterogeneity are emerging; however, standardisation remains challenging. For example, many guidelines exist for OM diagnosis and treatment, but are usually region specific. A recent publication on OME management describes an international consensus [144], but the variability of risk of long-term sequelae and availability of resources for diagnosis and treatment makes international conformity difficult. Furthermore, even where guidelines exist (and physicians self-describe as guideline compliant) management and treatment often do not adhere to the guidelines, and are instead more often selected based on the preferences of the service provider [145]. Similarly, a systematic review of antibiotic and analgesic prescriptions concluded that the introduction of a national guideline had only a modest effect on prescription appropriateness [146].

Impacts of heterogeneity in study design, OM case definitions and reported bacterial aetiology was highlighted in a 2016 epidemiological review of OM in developing countries which found difficulty in drawing generalisations from internationally sourced data due to methodological heterogeneity between studies [147]. Variation in microbiological reporting was also an identified limit of a systematic review of OM microbiome data among First Nation populations [148]. Microbiological heterogeneity is being addressed through establishment of recommended procedures. One example is the World Health Organization recommendations for *S. pneumoniae* detection [149] that were designed to reduce variability in *S. pneumoniae* vaccine trials. These recommendations can be applied in NP carriage studies and provide a standardised method for collecting specimens from discharging ears.

Similar recommendations for other otopathogens and the wider OM microbiome are lacking. Addressing this gap is important for future microbiome meta-analyses that will be limited without improved methodological standardisation between studies.

IMPLICATIONS FOR CLINICAL PRACTICE AND FUTURE RESEARCH GOALS

Key questions remain about microbiomic dynamics in OM pathogenesis—

While recent research has revealed new knowledge about the complexity of the OM microbiome, much remains unknown about the specific roles of the URT and middle ear microbiota in OM pathogenesis and treatment responses. The microbiome of the middle ear, both in health and disease, remains poorly defined. Large studies are needed to determine the normal variation of middle ear microbiota among healthy individuals, as well as the degree of microbiota variation between OM patients. Understanding variation among patients with similar OM diagnoses may be essential to determining whether microbial endotyping approaches could be used to inform precision medicine approaches to care, similar to the approaches being applied in lower respiratory contexts (e.g. [150]). Key contexts where there is a current scarcity of data include the development of the URT microbiome among children with early-onset OM (before 3–6 months of age) and in populations with high cross-sectional CSOM prevalence. CSOM can be challenging to treat and treatment failure rates are typically high (e.g. 70% treatment failure rate among Aboriginal and Torres Strait Islander children with CSOM treated with 6–8 weeks of topical ciprofloxacin or topical framycetin-gramicidin-dexamethasone [151]). Understanding the role of the middle ear microbiome in CSOM pathogenesis and treatment failure remains an important research goal.

Improved middle ear sampling procedures are needed—The limitations of current sampling methods should be considered when reporting middle ear microbiome data. It may not be possible to prevent contact with the EAC when middle ear rinsing is used, and thus, interpretation of microbiome data from rinse specimens must consider whether detected taxa may have originated from the EAC. Testing of paired EAC specimens (collected prior to myringotomy) and middle ear specimens may aid data interpretation; however, optimal analytic strategies for distinguishing contaminating EAC taxa in middle ear microbiome data remain to be determined. Current sampling limitations may be overcome using methods that avoid contact with the EAC or outer surface of the tympanic membrane. For example, retractable sheathed swabs may be a useful alternative to middle ear rinsing for microbiome studies. Determining the optimal middle ear sampling method for avoiding EAC contact is a priority research goal.

In the absence of protected sampling methods, we recommend recording and reporting of middle ear specimen quality as an essential first step to addressing this issue. For example, middle ear specimens used in microbiome analyses could be categorised as having either: no contact with EAC (e.g. direct aspiration from the middle ear; highest quality); brief contact with external surface of the tympanic membrane but not the EAC skin (moderate quality); brief contact with the EAC after spilling out of the middle ear during sampling (e.g. during a middle ear rinse; moderate to low quality); or prolonged contact with the EAC (e.g. sampling spontaneous discharges that have pooled in the canal space; lowest quality).

Dilution factors introduced by rinsing methods should also be quantified and reported, particularly in studies that aim to relate bacterial load measures to clinical parameters.

Research is needed to understand host factors that may shape the OM microbiome—Deeper understanding is needed about the impacts of host selective pressures on the development and trajectory of the URT and middle ear microbiomes, including systemic inflammation, immune responses and nutritional factors. Examples include studies to determine whether previously acquired antibodies (either from colonisation, infection or via maternal transfer) may influence the development of the infant URT microbiome; the impact of vaccines, particularly those targeting common otopathogens or respiratory viruses; host genetics; and potential modulation of systemic factors by the gut microbiome.

Meaningful translation of OM microbiome data—Currently, the most promising avenue for translating OM microbiome data into novel clinical prevention and/or treatment strategies is the potential for development of probiotic therapies. Recent URT microbiome studies suggest NP commensal taxa may have potential probiotic activity (e.g. *Corynebacterium* spp., *D. pigrum*), as may commensal species that are closely-related to otopathogens (e.g. *H. haemolyticus* isolates that produce a bacteriocin that inhibits NTHi *in vitro* [152]). Research of upper airway biomarkers associated with OM may also pave the way to new diagnostic tools with potential to inform personalised medicine [153].

Technical advances in microbiomic methods—Low-cost, high-throughput amplicon sequencing is likely to remain a widely used tool in OM studies, particularly for epidemiological studies that aim to relate clinical factors to broad shifts in URT and middle ear bacterial communities. Use of habitat-specific databases (e.g. eHOMD [106]) is expected to improve taxonomic resolution of short-read amplicons from OM specimens. Full-length 16S rRNA gene sequencing is also needed to improve taxonomic resolution; however, widespread uptake may be limited due to the higher costs associated with the sequencing technologies that support long-reads. Furthermore, it is not yet clear whether full-length 16S rRNA gene sequencing methods will have sufficient sensitivity (particularly for low abundance taxa) when applied to low biomass specimens.

Research applying other OMICs technologies is needed to advance the field; however, current methods may not be suited to low volume and low biomass specimens that may contain a high proportion of host-derived cells and molecules. For example, metagenomic sequencing has potential to provide deep characterisation of mucosal microbiomes, including: strain-level bacterial community profiling; characterisation of the mycobiome and virome (both eukaryotic viruses and phages); and resistome analyses that are important to understanding microbiomic responses to antibiotic pressures. However, successful application of metagenomic sequencing to OM specimen types requires technical advances to reduce signal from human DNA. Methods to selectively deplete human DNA from respiratory specimens have been developed [154], but most require fresh specimens and have not yet been validated for potentially low biomass OM specimens. Similar challenges may restrict future transcriptomic, proteomic and metabolomic studies of the OM microbiome.

Advancing beyond catalogues of microbes—Laboratory-based mechanistic studies are needed to advance understanding of the role of microbe-microbe and host-microbe interactions in OM pathogenesis and treatment responses. Such studies should include assessment of the impact of microecological conditions (e.g. temperature, pH, oxygen, heme availability), bacterial growth modes (e.g. biofilm, L-forms, intra-cellular persistence) and host environmental exposures (e.g. smoke exposure, vitamin D). The use of primary middle ear epithelial cells from humans (HMEEC) is showing promise as a relevant tool for OM research [155]. These HMEEC cultures have been shown to increase mucin secretion (Muc5B) in response to NTHi challenge, which is a key host response that contributes to OM pathogenesis *in vivo* [155]. Cell culture models such as the primary HMEEC may also be useful in the development of therapies to treat and prevent OM. In addition, existing human colonisation models, such as the pneumococcal [156] and NTHi [124] human challenge models, are expected to enable impacts of otopathogen colonisation on the healthy host microbiome to be elucidated, and allow assessment of interventions for restoring the microbiome following perturbation.

Improved inter-study standardisation is essential to advancing the field—Methodological heterogeneity is a barrier to meta-analyses of microbiomic data [157]. This limitation is well-recognised in the fields of environmental (e.g. [158]) and gut (e.g. [159]) microbiomics and has led to internationally standardised protocols. One example is the International Human Microbiome Standards project (IHMS; www.microbiome-standards.org) that provides standard operating procedures and recommends minimum reporting criteria for human gut microbiome studies. A similar framework is critically needed for OM microbiome studies, but has not yet been applied. One barrier to improving standardisation among OM microbiome studies is that data informing best practice are often lacking (e.g. optimal middle ear sampling methods, DNA extraction methods suitable for low volume specimens etc) or vary across populations (e.g. OM diagnostic guidelines). At a minimum, criteria used to define the middle ear diagnosis should be reported by all OM microbiome studies. Further research comparing optimisation and validation of sampling methods, laboratory and bioinformatic methods specifically for OM specimens is needed to define best practice within a standardised OM microbiome framework. In the absence of an accepted standardised framework, we recommend that minimum reporting criteria for OM microbiome studies are adopted (Box 3).

Conclusion

Recent advances have expanded understanding about the types of bacteria in the URT that may directly or indirectly contribute to OM pathogenesis. There is vast opportunity to extend this work to middle ear specimens; however, technical advances are needed to overcome limitations related to low specimen volume/biomass and high relative proportions of host cells. Further studies are needed to expand understanding of host and environmental pressures that may shape the OM microbiome, and to determine the mechanisms that underlie the microbe-microbe relationships suggested by DNA-based microbiomic studies. Standardisation of sampling and analytic methods is critically needed to advance the field and will be essential to future microbiomic meta-analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1:**Summary of OMICs technologies used to study the otitis media microbiome**

Amplicon sequencing	DNA sequencing targeted at ubiquitous genes that contain taxon-specific variation. Bacterial community (or microbiota) profiling by amplicon sequencing of the 16S rRNA gene is the most commonly used microbiomic method in otitis media studies. Important limitations of short-read amplicon sequencing (where only a portion of the 16S rRNA gene is sequenced) include low resolving power (i.e. may not identify bacteria to the species-level) and distortion of relative abundance profiles due to amplification biases.
Mycobiomics	An amplicon sequencing method that is used to characterise fungal communities.
Metagenomics	Shotgun sequencing of all DNA in a specimen. An advantage of metagenomic sequencing is that it can provide strain-level identification of bacteria, as well as the genomes of other microbes that may be present (e.g. viruses and fungi). Metagenomics will provide the most comprehensive assessment of the genetic potential of a microbial community; however, analysis of otitis media specimen types can be challenging due to a high proportion of reads (>90%) mapping to human DNA [63]. Methods to selectively exclude human DNA are available [154], but have not yet been validated for otitis media specimen types. Additionally, no RNA-based viruses can be identified with this method.
Transcriptomics	A RNA-based measure of all genes expressed in a sample. A small number of studies have used transcriptomics to study human gene expression in otitis media (e.g. [160]). Pathogen transcriptomes were reported by one study of nasopharyngeal aspirates [71].
Proteomics	A measure of all proteins in a specimen. One study has used proteomics to investigate innate immune responses in middle ear fluid from patients with chronic otitis media [63]. Assaying microbial proteomes directly from specimens may be challenging when a high proportion of the proteome is derived from host-produced molecules.
Metabolomics	A measure of all metabolites in a sample. Metabolomics is an emerging technology with potential respiratory diagnostic applications [161]. Metabolomic studies of otitis media specimens are emerging [153], but there are not yet any published data. As with other OMICs technologies, distinguishing microbial-specific signal may be difficult when high proportions of host-derived molecules are present.

Box 2:**Abbreviations used**

25OHD	25 hydroxy vitamin D
AOM	Acute otitis media
ARI	Acute respiratory infections
CoNS	Coagulase-negative Staphylococcus
CSOM	Chronic suppurative otitis media
EAC	External auditory canal
LGG	<i>Lactobacillus rhamnosus</i> GG
LME	Linear mixed effect model
MEF	Middle ear fluid
NP	Nasopharyngeal
NTHi	Non-typeable <i>Haemophilus influenzae</i>
OM	Otitis media
OME	Otitis media with effusion
OTUs	Operational taxonomic units
PCV	Pneumococcal conjugate vaccine
rAOM	Recurrent acute otitis media
RES	Relative effect size
URT	Upper respiratory tract

Box 3:**Recommended minimum reporting criteria for OM microbiome studies**

- Minimum demographic data should include the OM diagnosis, cohort age and details of any intervention exposures (where relevant).
- Criteria used to diagnose OM should be reported. Guidelines used to define diagnostic criteria should be cited.
- Procedures used to sample the URT and middle ear should be detailed.
- Specimen quality should be assessed and reported. For middle ear specimens, quality criteria describing the degree of specimen contact with the external auditory canal should be defined and reported.
- Procedures used to maintain specimen integrity should be reported, especially where RNA or other highly reactive molecules are to be assayed.
- For DNA sequencing-based studies, bacterial load should be quantified and reported.
- DNA extraction methods should be reported in detail.
- Primers and conditions used to prepare sequencing libraries should be reported.
- The sequencing platform and analytic pipeline should be reported, including details of any quality criteria used to filter sequence data.
- Methods used to identify and/or exclude confounding due to residual nucleic acid contamination in sterile laboratory reagents and kits should be described.
- The types of negative and positive controls used should be reported, including reporting whether negative controls were sequenced. Results of negative and positive controls should be reported, either within the main manuscript or in a supplementary file. Negative control sequence data should also be included when uploading datasets to public repositories.
- Where possible, the bioinformatic code used to analyse microbiomic data should be included as a supplementary file.

Taxa reported by microbiomic studies of external auditory canal and middle ear specimens

Table 1:

Diagnosis Number of studies References Bacteria	EAC Controls, OME, AOM	Middle ear specimens				Chronic perforations ^d	AOM-TT or PFTO
		OME/COM ^A	OME/rAOM ^B	AOM/rAOM/AOMwIP ^C			
	3	5	1	3	3	2	
	[4,6,64]	[2-4,7,18]	[21]	[6,61,62]	[9,11,20]	[5,17]	
	<i>Alloiococcus</i> (2)	<i>Alcaligenaceae</i> (1)	<i>Alloiococcus</i> (1)	<i>Acinetobacter</i> (1)	<i>Alcaligenaceae</i> (1)	<i>Aerococcus</i> (1)	
	<i>Corynebacterium</i> (2)	<i>Alloiococcus</i> (5)	<i>Anaerococcus</i> (1)	<i>Actinobacteria</i> ^f (1)	<i>Alloiococcus</i> (2)	<i>Campylobacter</i> (1)	
	<i>Enterobacteria</i> (1)	<i>Caulobacterium</i> ^e (1)	<i>Brevibacterium</i> (1)	<i>Alloiococcus</i> (2)	<i>Anaerococcus</i> (1)	<i>Corynebacterium</i> (2)	
	<i>Haemophilus</i> (2)	<i>Chitinophagaceae</i> (1)	<i>Clostridiales</i> ^g (1)	<i>Corynebacterium</i> (2)	<i>Bacteroides</i> (1)	<i>Delftia</i> (1)	
	<i>Moraxella</i> (1)	<i>Corynebacterium</i> (3)	<i>Corynebacterium</i> (1)	<i>Haemophilus</i> (3)	<i>Brevibacterium</i> (1)	<i>Dolosigranulum</i> (1)	
	<i>Pseudomonas</i> (1)	<i>Enterobacteria</i> (3)	<i>Dolosigranulum</i> (1)	<i>Moraxella</i> (3)	<i>Campylobacter</i> (1)	<i>Eikenella</i> (1)	
	<i>Streptococcus</i> (2)	<i>Fusobacteria</i> ^f (1)	<i>Finegoldia</i> (1)	<i>Lactococcus</i> (1)	<i>Corynebacterium</i> (3)	<i>Enterococcus</i> (1)	
	<i>Staphylococcus</i> (2)	<i>Fusobacterium</i> (1)	<i>Haemophilus</i> (1)	<i>Prevotella</i> (1)	<i>Fusobacterium</i> (1)	<i>Haemophilus</i> (1)	
	<i>Turicella</i> (1)	<i>Haemophilus</i> (5)	<i>Moraxella</i> (1)	<i>Pseudomonas</i> (1)	<i>Gordonia</i> (1)	<i>Klebsiella</i> (1)	
		<i>Moraxella</i> (4)	<i>Parvimonas</i> (1)	<i>Sphingobacteria</i> ^f (1)	<i>Haemophilus</i> (2)	<i>Marinobacter</i> (1)	
		<i>Neisseria</i> (1)	<i>Peptoniphilus</i> (1)	<i>Staphylococcus</i> (3)	<i>Klebsiella</i> (1)	<i>Moraxella</i> (1)	
		<i>Porphyromonas</i> (1)	<i>Porphyromonas</i> (1)	<i>Streptococcus</i> (3)	<i>Nevista</i> (1)	<i>Parvimonas</i> (1)	
		<i>Streptococcus</i> (4)	<i>Pseudoclavibacter</i> (1)	<i>Turicella</i> (3)	<i>Ochrobactrum</i> (1)	<i>Peptoniphilus</i> (1)	
		<i>Pseudomonas</i> (3)	<i>Pseudomonas</i> (1)	<i>Veillonella</i> (1)	<i>Oligella</i> (1)	<i>Peptostreptococcus</i> (1)	
		<i>Staphylococcus</i> (5)	<i>Serratia</i> (1)		<i>Parvimonas</i> (1)	<i>Porphyromonas</i> (1)	
		<i>Stenotrophomonas</i> (1)	<i>Staphylococcus</i> (1)		<i>Peptoniphilus</i> (1)	<i>Propionibacterium</i> (1)	
		<i>Turcibacter</i> (1)	<i>Streptococcus</i> (1)		<i>Pedimicrobium</i> (1)	<i>Pseudomonas</i> (2)	
		<i>Turicella</i> (3)	<i>Turicella</i> (1)		<i>Peptostreptococcus</i> (1)	<i>Serratia</i> (1)	
					<i>Porphyromonas</i> (1)	<i>Sporobacterium</i> (1)	
					<i>Propionibacterium</i> (1)	<i>Staphylococcus</i> (2)	
					<i>Proteus</i> (1)	<i>Streptococcus</i> (2)	
					<i>Pseudomonas</i> (2)	<i>Turicella</i> (1)	
					<i>Raoultella</i> (1)		
					<i>Shewanella</i> (1)		

Diagnosis	EAC Controls, OME, AOM	Middle ear specimens			AOM-TT or PTTO
		OME/COM ^A	OME/rAOM ^B	AOM/rAOM/AOMwIP ^C	
Number of studies	3	5	1	3	2
References	[4,6,64]	[2-4,7,18]	[21]	[6,61,62]	[5,17]
Fungi	<i>Aspergillus</i> (1) <i>Candida</i> (1) <i>Malassezia</i> (1) <i>Nakaseomyces</i> (1) <i>Penicillium</i> (1) <i>Saccharomyces</i> (1)			<i>Staphylococcus</i> (3) <i>Streptococcus</i> (1) <i>Turicella</i> (1) <i>Aspergillus</i> (1)	<i>Aspergillus</i> (1) <i>Candida</i> (1)

Data are limited to dominant taxa reported by each study. Unless otherwise indicated, dominant taxa are shown at the genus-level, consistent with nomenclature used in most microbiota studies. Bracketed numbers indicate the number of studies that reported each taxon. AOM: Acute otitis media. AOMwIP: Acute otitis media with spontaneous perforation of the tympanic membrane. AOM-TT: Acute otitis media associated with tympanostomy tubes with otorrhea. COM: Chronic otitis media. EAC: External auditory canal. OME: Otitis media with effusion. rAOM: Recurrent acute otitis media. PTTO: Post-tympanostomy tube otorrhea. References are numbered consistent with the main manuscript.

^a: Krueger et al. [2] defined COM as the presence of middle ear fluid for >3-months.

^b: Boers et al. [21] combined data from patients with OME or rAOM.

^c: Sillanpää et al. [61] combined data from patients with AOM, AOM with spontaneous perforation of the tympanic membrane (AOMwIP) or AOM associated with tympanostomy tubes complicated by otorrhea.

^d: Includes data from patients with suppurative and dry chronic perforations.

^e: Genus name as reported by Chan et al. [18].

^f: Sequences not identified below the phylum-level.

^g: Sequences not identified below the class-level

Table 2.

Advances towards therapeutics that use commensal species to prevent infection from related bacterial pathogens

Disease	Target pathogen	Commensal Species	Development stage	Ref
Otitis media	<i>Streptococcus pneumoniae</i>	α -haemolytic <i>Streptococci</i>	Phase II -children	[162]
Pharyngitis	<i>Streptococcus pyogenes</i>	<i>Streptococcus salivarius</i>	Licensed product	[163]
Pneumonia	<i>S. pneumoniae</i>	<i>Streptococcus mitis</i>	Pre-clinical	[164]
Meningitis	<i>Neisseria meningitidis</i>	<i>Neisseria lactamica</i>	Pre-clinical	[165,166]
Bacteraemia	<i>Staphylococcus aureus</i>	<i>Staphylococcus lugdunensis</i>	Phase I -adults	[114]
Salmonellosis	<i>Salmonella typhi</i> (murium)	<i>Escherichia coli</i>	Pre-clinical	[167]