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REVIEW

Translating Recent Microbiome Insights in Otitis Media into Probiotic Strategies

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SUMMARY The microbiota of the upper respiratory tract (URT) protects the host from bacterial pathogenic colonization by competing for adherence to epithelial cells and by immune response regulation that includes the activation of antimicrobial and (anti-)inflammatory components. However, environmental or host factors can modify the microbiota to an unstable community that predisposes the host to infection or inflammation. One of the URT diseases most often encountered in children is otitis media (OM). The role of pathogenic bacteria like *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in the pathogenesis of OM is well documented. Results from next-generation-sequencing (NGS) studies reveal other bacterial taxa involved in OM, such as *Turicella* and *Alloiococcus*. Such studies

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can also identify bacterial taxa that are potentially protective against URT infections, whose beneficial action needs to be substantiated in relevant experimental models and clinical trials. Of note, lactic acid bacteria (LAB) are members of the URT microbiota and associated with a URT ecosystem that is deemed healthy, based on NGS and some experimental and clinical studies. These observations have formed the basis of this review, in which we describe the current knowledge of the molecular and clinical potential of LAB in the URT, which is currently underexplored in microbiome and probiotic research.

KEYWORDS *Haemophilus influenzae*, *Lactobacillus*, microbiome, *Moraxella catarrhalis*, otitis media, probiotics, *Streptococcus pneumoniae*

INTRODUCTION

Various physical, chemical, and infectious agents can enter the human body via the upper airways, and this causes humans to be prone to upper respiratory tract (URT) diseases. The URT consists of the anterior nares, nasal passages, paranasal sinuses, the nasopharynx and oropharynx, and the portion of the larynx above the vocal cords (1). In children, the most common URT infection is otitis media (OM). OM encompasses a spectrum of disease conditions characterized by accumulation of fluid in the middle ear cavity and inflammation of the middle ear cleft (Fig. 1).

OTITIS MEDIA

Risk Factors for Otitis Media

A common pathway to all forms of OM is impaired function of the eustachian tube and inflammation of the middle ear (2–4). This is illustrated by the increased prevalence of middle ear effusion in children with an inherent anatomical abnormality causing dysfunction of the muscles involved in eustachian tube opening, such as children with cleft palate or Down syndrome (5, 6). Eustachian tube dysfunction may also be caused by congestion and inflammation of the mucosal lining (e.g., following an URT infection) or by mechanical obstruction from enlarged adenoids (7). As a consequence, accumulation of middle ear fluid can occur, creating an ideal environment for bacterial growth and the development of inflammation.

As it is a multifactorial condition, anatomical, host-related, and environmental factors play a role in OM (Fig. 2) (3, 8). Host factors that increase the risk for OM are, for instance, younger age, genetic predisposition, race and ethnicity, immunodeficiency, and laryngopharyngeal reflux. Environmental factors that have a negative influence on OM, on the other hand, are winter season, formula feeding or limited breastfeeding, exposure to cigarette smoke, low socioeconomic status, presence of older siblings, day care attendance, and pacifier use (as reviewed by Schilder et al. [3]). Although many factors are thus suggested to be involved, the pathogenesis is not yet fully understood.

Different Forms of Otitis Media and Their Incidences

Different forms can be distinguished in OM. In this review, the widely used definitions as defined by Bluestone and by Schilder et al. (3, 9) are used (Table 1). Acute OM (AOM) is generally defined as the rapid onset of acute infection within the middle ear, characterized by signs and symptoms such as otalgia and fever. Otitis media with effusion (OME) is characterized by inflammation of the middle ear without signs or symptoms of acute infection and accompanied by the accumulation of fluid. Middle ear effusion (middle ear fluid [MEF]) is a liquid in the middle ear which may be serous, mucoid, or purulent. The duration of the effusion may range from less than 3 weeks (acute) to 3 weeks up to 2 to 3 months (subacute) or more than 3 months (chronic). Fluid in OME may persist in the middle ear cavity following an episode of AOM or result from eustachian tube dysfunction caused by a URT infection. It is the most common cause of hearing impairment in childhood, and resolution of hearing loss is the main treatment goal for OME (10). Chronic otitis media (COM) is defined as chronic inflammation ($\geq$3 months) of the mucosa and submucosa of the middle ear and may result in changes not only to the mucosa and submucosa but also to the tympanic membrane

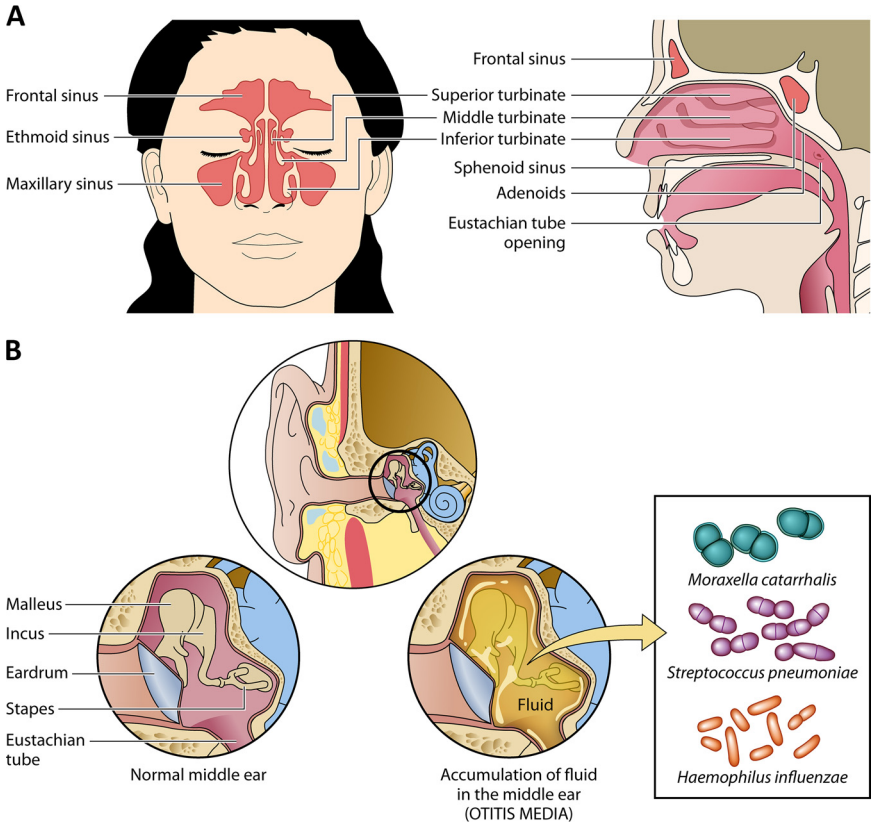

FIG 1 Anatomy of the nasal cavity and characteristics of otitis media (OM). (A) Anatomy of the nasal cavity, depicting the connections between the different niches. (B) In healthy conditions, the middle ear is filled with air, while OM is characterized by the presence of fluid in the middle ear and the inflammation of the middle ear cleft. Dysfunction of the eustachian tube prevents the middle ear fluid from draining normally and, thus, creates an ideal environment for bacterial growth and the development of inflammation. The three main pathogens in (A)OM are *Moraxella catarrhalis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, but other pathogens are emerging, such as *Turicella* and *Alloiococcus*, especially for more chronic forms of OM (see the text).

(e.g., chronic suppurative otitis media [CSOM]) and ossicles. COM is the most severe form of OM but is very uncommon in developed countries (11).

Most children experience at least one episode of AOM (12), with a peak period of occurrence between 6 and 12 months. Recent monitoring data indicate that 46% of U.S.

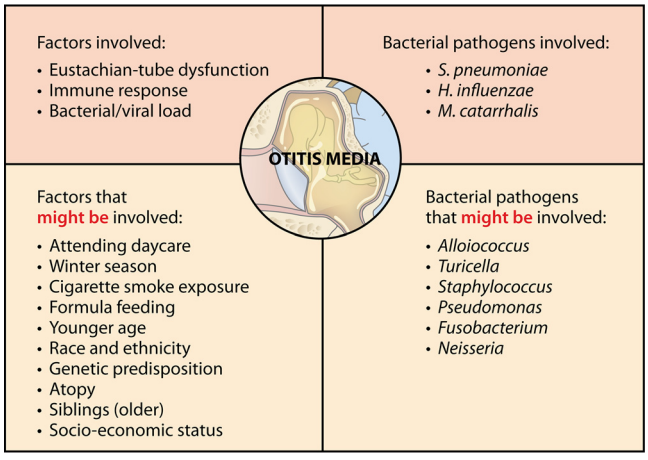

FIG 2 Factors and pathogens involved in OM pathogenesis. Based on data from Rovers et al. and Schilder et al. (3, 12) and the data presented in Table 2.

TABLE 1 Overview of different types of OM and their definitions

OM type	Definition[a]
Otitis media (OM)	A spectrum of disease conditions characterized by accumulation of fluid in the middle ear cavity and inflammation of the middle ear cleft
Acute otitis media (AOM)	The rapid onset of acute infection within the middle ear, characterized by signs and symptoms such as otalgia and fever
Otitis media with effusion (OME)	Inflammation of the middle ear without signs or symptoms of acute infection and accompanied by accumulation of fluid
Chronic otitis media (COM)	Chronic inflammation (≥3 mos) of the mucosa and submucosa of the middle ear; may result in changes not only to the mucosa and submucosa but also to the tympanic membrane and ossicles
Chronic suppurative otitis media (CSOM)	Chronic inflammation (≥3 mos) of the middle ear and mastoid mucosa with a nonintact tympanic membrane (perforation or ventilation tube) and persistent ear discharge

[a]The definitions are from references 3 and 9.

children have already suffered at least one episode of AOM before their first birthday (13). Typically in OME, a bimodal distribution in prevalence occurs, with a first peak around 2 years and a second peak around 5 years of age (14). It represents the most common form of OM in young children, with a point prevalence of ca. 20% (12).

Antibiotics in Otitis Media

In childhood, OM is a leading cause of antibiotic prescription (15). The rates of antibiotic prescription for AOM vary from 56% in the Netherlands to 95% in the United States (16, 17). However, contrary to what these numbers suggest, clinical practice guidelines first recommend a focus on pain relief without prescribing antibiotics, since spontaneous healing without complications is often observed and antibiotics only have a slight effect on pain in AOM (18). Depending on the age of the child and the severity of symptoms, however, antibiotics may be indicated to treat AOM according to published guidelines (4). As recently shown by a Cochrane Review (19), the use of oral antibiotics to treat OME has been associated with both benefits and harms, since it is associated with an increased chance of complete resolution at various follow-up times but these children are more likely to experience side effects like diarrhea, vomiting, or skin rash. Furthermore, the impact of antibiotics on hearing is unclear and there is no evidence that antibiotics are associated with fewer ventilation tube insertions.

Microbial Etiology of Otitis Media

Both viruses and bacteria are implicated in the pathogenesis of AOM; however, less is known about fungi. In children between 6 months and 3 years of age, about 90% of episodes of AOM are associated with a viral URT infection (20–22). The resulting inflammation of the epithelium in the nasopharynx and eustachian tube creates a negative middle ear pressure and promotes movement of bacteria and/or viruses into the middle ear, where they can cause infection. The risk of developing AOM after a viral URT infection has been related to the number of pathogens colonizing the nasopharynx. Half of the children carrying the three main AOM pathogens, *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis*, develop AOM after a viral URT infection, compared to only 10% if none of these pathogens are present (23). The degrees of dominance of these otopathogens during OM have undergone dynamic changes since the introduction of the pneumococcal conjugate vaccines. A drop in the detection of *S. pneumoniae* was observed, while there appears to be an increase in the prevalence of *M. catarrhalis*. *H. influenzae*, however, appears to remain a dominant pathogen (24).

Via culture-dependent data, *S. pneumoniae*, NTHi and *M. catarrhalis* have long been described as the three main pathogens related to all other forms of OM as well (12), but next-generation-sequencing (NGS) approaches where the microbiome in diseased subjects is compared with the microbiome in healthy subjects have recently highlighted that other bacteria can be involved, as discussed in the next paragraphs. Viruses, commonly detected via immunological and molecular techniques, also play a role in OM. Studies indicate that the influenza A virus (20), respiratory syncytial virus

(25), human rhinovirus (26), and adenovirus (20) could predispose to bacterial infection in AOM. These viruses can create changes in eustachian tube functioning by initiating inflammation (27), altering the biochemical and rheological properties of airway mucus (22), and compromising the mucociliary clearance (22, 28). Furthermore, by upregulating the expression of eukaryotic receptors, viruses can increase bacterial adherence and colonization (22, 29). To map the community of viruses and bacteriophages (i.e., the virome) via NGS approaches, standard 16S rRNA gene amplicon sequencing is not appropriate. Shotgun sequencing, dedicated DNA extraction, and other related protocols are needed, and these approaches are less widely adopted. Recently, the human respiratory virome is gaining more interest (30–35); however, to the best of our knowledge, no metagenomic URT virome data are yet available for children suffering from OM. Similarly, little is documented about the community of URT fungi (i.e., mycobiome) present during OM. Similar to the situation for the virome, different sequencing methods are needed to investigate the mycobiome, such as targeting of the internal transcribed spacer (ITS) regions of the rRNA locus for sequencing (36). Unfortunately, again, no mycobiome data are available for OM. Presumably if viruses or fungi were the primary cause of the infection, they would have been identified already. It is reasonable, however, that some specific viruses or fungi interact with the important pathogens to facilitate their infection and have always been underestimated. There is thus a clear need for more dedicated metagenomic studies that will give a better global overview of the total URT microbial community (bacteria, viruses, bacteriophages, and fungi). Such knowledge might be interesting for new therapies, as targeting important bystanders or cofactors might help to resolve the disease.

Since the information about the URT virome and mycobiome is limited, we will focus in this review on the bacterial microbiome and the potential bacterial interactions between OM pathogens and beneficial bacteria.

THE BACTERIAL MICROBIOME OF OTITIS MEDIA PATIENTS

The relationship between bacterial community composition in the URT, risk of pathogen colonization, and OM symptoms is increasingly being studied via culture-independent approaches like NGS, which is currently the main technique used for investigating microbial communities. NGS approaches certainly have their limitations in rather low-biomass niches like the respiratory tract, including the presence of inhibitors and contaminants, the difficulty in discriminating between live and dead bacteria, the short read lengths, and the lack of information about viruses and fungi and about absolute microbial numbers. However, these culture-independent approaches have still revealed novel insights on potential pathogenic and beneficial bacteria, as will be discussed below. It should be noted, however, that most approaches only identify the bacteria on the genus level, while pathogenicity is expressed at the strain level. This makes the distinction between commensal and potentially pathogenic species challenging. Furthermore, inconsistencies in microbiome studies can be due to differences in disease parameters, geographical location (37), sampling, storage, DNA extraction (38), sequencing approach (e.g., the targeting of different hypervariable regions of the 16S rRNA gene, indicated with V plus a number), and bioinformatic analysis (Table 2), among others, that can all favor and/or underestimate certain species. The next paragraphs aim to map the current knowledge about the bacterial microbiome differences between AOM, OME, and COM.

Development of the Healthy URT Microbiome in Children

The microbiome of the URT is variable over time and depends on several, often environmental factors (Fig. 3) (1, 39). As the nose and nasopharynx are interconnected with the middle ear cavity, the microbiota of these niches can influence the middle ear microbiota (Fig. 1) and will be discussed in this paragraph as well. Already after 1 day of life, Bosch et al. (40) observed that the URT microbiota shifts to a *Streptococcus viridans*-predominated profile. After 6 months, a change toward a *Corynebacterium pseudodiphteriticum/propinquum*-, *Dolosigranulum pigrum*-, *M.*

TABLE 2 Overview of some of the most pioneering URT and OM microbiome studies

Focus or OM type	Sample type(s)	Disease-associated bacteria[a]	Sequencing method	No. of subjects (country)	Age(s) (mos or as indicated), no. of subjects/group	Reference
Focus						
Respiratory microbiota in healthy infants	Nasal swabs	*Haemophilus*, *Streptococcus*	454 sequencing, V5-V7 region	60 (The Netherlands)	1.5–24	41
	Nasal aspirates	*Moraxella*, *Streptococcus*, *Haemophilus*	Illumina MiSeq, V4 region	234 (Australia)	2–12	52
OM type						
AOM	Nasal swabs	*S. pneumoniae*, *Haemophilus*, *Rothia*, *Actinomyces*	454 sequencing, V1-V2 region	108 (USA)	6–78	59
	Nasal swabs	*Moraxellaceae*, *Streptococcaceae*, *Pasteurellaceae*	454 sequencing, V3-V5 region	153 (Switzerland)	0–24	60
	Nasal swabs	*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*	454 sequencing, V1-V2 region	240 (USA)	3–36	15
	MEF aspirates	*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *T. otitidis*, *S. auricularis*	Illumina MiSeq, V4 region	79 (Finland)	5–42	61
	Nasal swabs	*Moraxella*, *Haemophilus*, *Streptococcus*	Illumina MiSeq, V4 region	139 (USA)	1–12	282
	MEF aspirates, middle ear aspirates, ear canal swabs, nasal swabs	*Haemophilus*, *Turicella*, *Alloiococcus*, *Staphylococcus*	Illumina MiSeq, V3-V4 region	196 (Australia)	0–60	64
OME	MEF aspirates, adenoid and tonsil tissue samples	*Pseudomonadaceae*, *Streptococcaceae*, *Fusobacteriaceae*, *Pasteurellaceae*	454 sequencing, V3-V4 region	1 (USA)	96	65
	MEF aspirates, adenoid swabs, nasal swabs	*Alloiococcus*, *Haemophilus*, *Streptococcus*, *Moraxella*	Illumina MiSeq, V1-V3 region	11 (Australia)	3–10 yrs	66
	MEF aspirates, adenoid swabs	*Alloiococcus*, *Haemophilus*, *Moraxella*, *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Corynebacterium*	Illumina MiSeq, V3-V4 region	18 (Australia)	1–16 yrs	67, 68
	Middle ear, adenoid, and tonsil swabs	*Fusobacterium*, *Haemophilus*, *Neisseria*, *Porphyromonas*	Illumina MiSeq, V3-V4 region	10 (New Zealand)	2–10 yrs	70
COM	Middle ear swabs, mastoid swabs	*Haemophilus*, *Staphylococcus*, *Alloiococcus*	Illumina MiSeq, V3-V4 region	46 (New Zealand)	6 mos–87 yrs	72
	MEF aspirates	*Haemophilus*, *Moraxella*, *Turicella*	Illumina MiSeq, V4 region	55 (USA)	0–24, 25 subjects; >24, 30 subjects	71

[a]Bacteria were significantly more abundant in this group, as indicated by the authors.

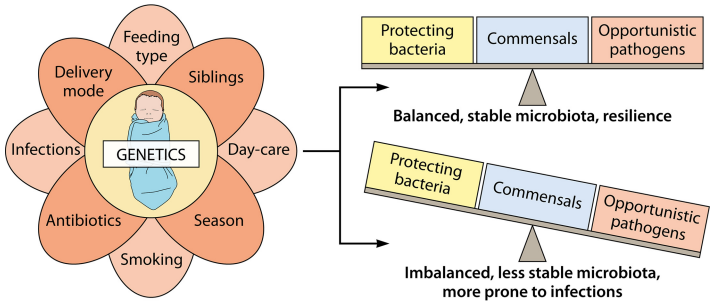

FIG 3 Factors influencing the respiratory microbiota and/or bacterial density. First colonization in early life takes place during birth. The mode of delivery (natural versus Caesarian section) largely influences the microbial community in the newborn's respiratory tract. Afterwards, the dynamics and evoluation of the microbiota are driven by many other environmental factors, such as feeding type, having older siblings or not, attending day care, the season, growing up in an environment with smokers, taking antibiotics, and having infections. Together with the host's genetics, which influences the bacterial density in the nasopharynx, the microbiota can develop toward a balanced, stable microbiota where resilience, i.e., the ability of the host to remain healthy even when exposed to a stress, occurs. Conversely, the microbiota can also develop toward a community that is imbalanced, less stable, and more prone to infections and inflammation. The figure is based on data from references 1, 3, 15, 42, and 52.

catarrhalis/nonliquefaciens-, *S. pneumoniae*-, and *H. influenzae*-dominated community or a mixed community with these bacteria was observed. In total, 11 nasopharynx microbiota profiles (termed clusters) were identified using Illumina MiSeq sequencing (V4 region), which confirmed earlier results (41). Biesbroek et al. (41) also noticed associations between certain taxa and microbiota stability during the first 2 years of life. Less-stable profiles contained a high abundance of *Haemophilus* and *Streptococcus*. In contrast, an early presence and high abundance of *Moraxella* and *Corynebacterium*/*Dolosigranulum* in the first period of life was associated with a more stable pattern (41), which was confirmed later on by the same researchers (40, 42) using Illumina MiSeq sequencing (V4 region) as well. In addition, Santee and colleagues (43), using a 16S rRNA PhyloChip sequencing approach focusing on the V5 region, observed an association between an enrichment of *Moraxella nonliquefaciens* in the nasopharynx of American children and acute sinusitis. The facts that, on one hand, early colonization of *Moraxella* is associated with a stable microbial pattern and, on the other hand, *M. nonliquefaciens* is enriched in children suffering from acute sinusitis highlights that association with health and disease should be studied at the strain or species level, since different strains and species have different virulence characteristics (44). This is not always possible with the currently available NGS approaches, especially not with amplicon sequencing, although pipelines such as the Divisive Amplicon Denoising Algorithm 2 (DADA2) that take into account genuine amplicon sequence variants (45) and shotgun sequencing approaches are an important step forward. Moreover, full-gene 16S rRNA gene sequencing analysis on the PacBio system could provide microbiome data at the species level in future microbiome analysis (46).

As already mentioned, host and environmental factors play an important role in the maturation of the URT microbiome (Fig. 3). First of all, the mode of delivery seems to have a significant effect on the URT microbiota directly after birth. Indeed, a longitudinal study organized in the United States by Bosch et al. (40) has followed 102 children in the first 6 months of life and analyzed the bacterial DNA from nasopharyngeal swabs via Illumina MiSeq sequencing of the V4 variable region of the 16S rRNA gene. These authors observed that children who were delivered vaginally versus by Caesarean section carried a URT microbiota resembling, respectively, the maternal vaginal or skin microbiota directly after birth. This study confirmed earlier observations about the relationship between the mode of delivery and the baby's microbiota by Dominguez-Bello et al. (47), where babies were only sampled immediately after birth and their microbiota was compared with the microbiota of different niches of the mother's body

via 454 pyrosequencing of the V2 variable region. Children born by Caesarian section showed diminished colonization with commensals like *Corynebacterium* and *Dolosigranulum* (40). The latter result was also observed in children with limited breast feeding (42). The members of the *Dolosigranulum* genus are rather unexplored lactic acid bacteria (LAB) belonging to the family of *Carnobacteriaceae*, while the *Corynebacterium* genus includes pathogenic species which are involved in diseases like diphtheria (48) and pneumonia (49), as documented for skin commensals with an inflammatory potential depending on the context (50). Both *Dolosigranulum* and *Corynebacterium* are gaining more interest recently, as they seem to be prevalent members in the nose and nasopharynx microbiota of healthy adults (51).

Next to the mode of delivery and feeding type, antibiotic use, host genetics, season, cohabiting with siblings, antibiotic use, attending day care, and exposure to cigarette smoke have an influence on the microbiome of children as well (15, 52–57). However, only a small number of studies with different sampling and sequencing methods have been performed on these topics.

The Bacterial Microbiome in Acute Otitis Media (AOM)

Taking the influence of all these (environmental) factors described above into account, it is not surprising that the URT microbiota balance can be easily disturbed, resulting in health issues such as OM. Investigations into the relationship between the microbiota of the middle ear and OM are, however, encountering some limitations, since it is difficult to obtain clinical samples from healthy control subjects, as getting access to the middle ear is only ethical when medical problems occur. Considering the fact that several URT niches are interconnected (Fig. 1) and these microbiotas can influence each other, the microbiome results investigated via the sampling of several of these URT niches are discussed in this section. Moreover, recent data indicate that microbiota composition in the nasopharynx could predict duration of AOM with tympanostomy tubes even better than MEF microbiota (58).

In one of the first NGS approaches on AOM, Laufer et al. (59) investigated nasal swabs of 108 children with and without AOM via 454 sequencing (V1-V2 region). The authors observed a relationship between the presence of *S. pneumoniae*, one of the main OM pathogens, and a less diverse (i.e., the number of different species in an environment) and less even (i.e., how close in population size each species in an environment is) microbial community. Furthermore, the presence of *Haemophilus*, *Rothia*, and *Actinomyces* was associated with an increased risk of AOM. In contrast, a potentially protective microbiota consisting of bacterial species such as *Corynebacterium*, *Dolosigranulum*, *Propionibacterium*, *Lactococcus*, and *Staphylococcus* was associated with a decreased risk of pneumococcal colonization and AOM. The same research group subsequently performed an analysis of nasal swabs of 240 children that also took the use of antibiotics in the 6 months before sampling into account (15). The mean levels of the AOM-associated taxa *Rothia* and *Actinomyces* were higher in children that received antibiotics in the past 6 months. Of interest for potential probiotic applications, *Lactococcus*, *Anoxybacillus*, and members of the family *Enterobacteriaceae* appeared negatively associated with colonization by each of the three classical bacterial AOM pathogens *M. catarrhalis*, *S. pneumoniae*, and *H. influenzae* and with AOM in children who used antibiotics in the past 6 months (15). However, such an association does not necessarily imply a causal relation between these potential probiotic taxa and health. Therefore, additional experimental evidence is necessary, as will be further discussed below in more detail. Hilty et al. (60) observed that the nasopharyngeal microbiota of children suffering from AOM more frequently contained bacteria from the families of *Moraxellaceae*, *Streptococcaceae*, and *Pasteurellaceae*, in agreement with the three major AOM pathogens. Although it is impossible to discuss pathogenicity and beneficial properties at the family level, these taxa are known to contain many common URT pathogens. In contrast, taxa which potentially contain more beneficial commensals, such as *Staphylococcaceae*, *Flavobacteriaceae*, *Carnobacteriaceae*, and *Comamonadaceae*, were less prevalent in AOM patients than in the control children (Table 2).

In addition to the nasal and nasopharyngeal microbiota obtained via swab sampling, middle ear fluid (MEF) is also a specimen of interest for detailed microbiome analyses. Sillanpää et al. (61) investigated 90 MEF samples of 79 children between 5 and 42 months of age using a combination of nested PCR and Illumina MiSeq 16S rRNA gene amplicon sequencing (V4 region) and operational taxonomic unit (OTU) clustering. They observed dominance of *S. pneumoniae* in 14 samples (16%), *H. influenzae* in 15 (17%), and *M. catarrhalis* in 5 (5.6%), while the less well-known AOM pathogens *Turicella otitidis* and *Staphylococcus auricularis* dominated in two subjects each. For comparison, based on culture-dependent data, *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* were the pathogens detected in 22%, 19%, and 10% of the cases, respectively. This study thus showed that both culture-dependent and -independent techniques confirm that the three major AOM pathogens dominate MEF of children suffering from AOM but NGS can also point toward other emerging pathogens. *T. otitidis* and *Alloiococcus otitidis* are examples of such emerging pathogens. In the study of Sillanpää et al. (61), they were found in 5 (5.6%) and 3 (3.3%) MEF samples, respectively. Before, these pathogens were only occasionally reported to occur in AOM based on culture-dependent data (62, 63), but microbiome-based data revealing their relative abundance in OM patient samples are increasing. Also, in a more recent microbiome case-control study, *T. otitidis* and *A. otitidis* were detected in high abundances in the middle ear (relative abundances of 6.72% and 49.84% in MEF, respectively) and ear canal (relative abundances of 13.06% and 53.62%, respectively) of recurrent AOM patients (64). It should be noted, however, that this study used nasopharyngeal swabs of healthy controls to compare with and the relative abundances of both potential pathogens were very low in these nasal swab samples of both AOM patients and healthy controls. So although the study also identified *T. otitidis* and *A. otitidis* as emerging OM pathogens, it could not rule out the possibility of these strains belonging to the normal aural microbiota due to the high relative abundances in the ear canal.

The Bacterial Microbiome in OME and COM

Although less frequently than AOM, otitis media with effusion (OME) and chronic OM (COM) are also being characterized by NGS (Table 2). In one of the first studies in the field, Liu et al. (65) investigated the microbiota of tonsil, adenoid, and middle ear fluid specimens of one patient with COM via 454 sequencing (V3-V4 region). The study group saw overlapping communities in these three respiratory niches. The adenoids showed a more complex microbial profile, containing *Pseudomonadaceae*, *Streptococcaceae*, *Fusobacteriaceae*, and *Pasteurellaceae*, while the middle ear and tonsils were each dominated by just one family, *Pseudomonadaceae* and *Streptococcaceae*, respectively. This observation adds support to the assumption that the middle ear and the tonsil microbiota can originate from the adenoids (65). Subsequently, Jervis-Bardy et al. (66) provided a landmark study for OME, because they observed by Illumina MiSeq sequencing of the 16S rRNA V1-V3 region that OTUs from the classic AOM pathogens *Streptococcus*, *Haemophilus*, and *Moraxella* are also common in MEF, nasopharyngeal, and adenoid samples of 11 children with OME. Two follow-up studies also observed similarities between MEF and adenoids of OME patients (67, 68). However, an important difference from AOM appeared, namely, *A. otitidis* dominated the middle ear effusion microbiota (23% mean relative abundance), followed by *Haemophilus* (22%), *Staphylococcus* (11%), *Corynebacterium* (6%), *Moraxella* (5%), and *Streptococcus* (5%). These abundances were observed to be stable over time, as they did not change drastically after 1 year (68). Swabs of the adenoids, on the other hand, showed colonization by *Haemophilus* (25% mean relative abundance), *Moraxella* (14%), *Streptococcus* (13%), *Fusobacteria* (11%), and *Neisseria* (7%). *Alloiococcus* was inversely correlated with *Haemophilus*, found in greater relative abundance in unilateral effusion, and had a very low relative abundance in adenoid swabs ($<$1%) (67). In the external auditory canal, the same *Alloiococcus* was found to have the highest relative abundance (28%), followed by *Staphylococcus* (20.8%) and *Pseudomonas* (3.2%) (68). Thus, taken together, the current

data suggest that dominance of *A. otitidis* is associated with OME, while dominance of *M. catarrhalis*, *H. influenzae*, and *S. pneumoniae* may favor AOM. Furthermore, the studies of Chan and colleagues suggest that the external auditory canal and adenoids can both act as bacterial reservoirs for middle ear infections (67, 68). As perforations in the tympanic membrane sometimes occur in AOM, this can indeed give a free pass to bacteria that normally reside in the external auditory canal to move to the middle ear cavity (69). In contrast to the research discussed above, the study of Johnston et al. (70) did not reveal significant similarity between the microbiota of the adenoids and that of the middle ear in children with OME via microbial network analysis. Using Illumina MiSeq sequencing of the 16S rRNA V3-V4 region, the researchers observed higher relative abundances of *Haemophilus* and *Moraxella* in adenoid tissue than in the middle ear, where *Fusobacterium* and *Staphylococcus* were the most abundant genera. Across the adenoids, tonsils, and middle ear, however, *Fusobacterium*, *Haemophilus*, *Neisseria*, and *Porphyromonas* were the most abundant sequences. Furthermore, *Alloiococcus* and *Turicella* were only found in the middle ear samples, but the external auditory channel was not included in the study. Thus, no consensus exists about the adenoids being a source for OM pathogens, also called the "pathogen reservoir hypothesis."

With regard to chronic OM, 55 American children were sampled and 16S rRNA gene amplicon sequencing via the Illumina MiSeq Platform (V4 region) was performed, which resulted in different bacterial disease profiles. The six most abundant bacteria in the MEF samples of this study were *Haemophilus* (relative abundance 22.54%), *Moraxella* (11.11%), *Turicella* (7.84%), unclassified *Alcaligenaceae* (5.84%), *Pseudomonas* (5.40%), and *Alloiococcus* (5.08%), while *Streptococcus* accounted for 4.21% of the MEF bacterial reads (ranked as the 8th most abundant genus) (71). Neeff et al. (72) associated *Haemophilus*, *Staphylococcus*, and *Alloiococcus* with an increased risk of COM using Illumina MiSeq sequencing (V3-V4 region) in 24 patients with COM and 22 healthy controls. Higher relative abundances of *Novosphingobium*, *Staphylococcus*, *Escherichia-Shigella*, *Burkholderia*, and *Propionibacterium* were observed in the middle ear specimens of healthy controls.

Combination of NGS and (Translated) Koch's Postulates Can Identify New Pathogens or Probiotics

In 1890, Robert Koch published his four criteria to establish a causative relationship between a microbe (pathogen) and a disease (73). These postulates, although they have their limitations (74), had an enormous influence in medical microbiology. They state, among other things, that a pathogen should be isolated from a diseased organism and cause disease when introduced in a healthy organism. The latter point is quite important in the current era of NGS approaches to study bacterial communities in health and disease. Bacteria such as *A. otitidis* and *T. otitidis*, for example, are now gaining attention in the etiology of OM due to their high abundance in diseased children. However, because insights into their pathogenesis and molecular pathogenic characteristics are currently lacking, their role as pathogenic drivers of the disease is still under debate. On the other hand, microbiome insights indicate that the original Koch's postulates, which state that pathogens should not be found in healthy organisms, are not entirely valid for most opportunistic pathogens. Indeed, all OM pathogens, for example, can also be found in the URT of healthy persons, but generally in lower abundances (51).

Similarly, to identify new probiotic strains, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (75), knowledge about their increased prevalence and abundance in healthy persons is not sufficient. For this reason, we introduce possible translated "probiotic postulates," based on Koch's postulates, for the search for next-generation probiotics. These translated "probiotic postulates" are based on comparative microbiome research combined with experiments to determine a causative relationship with improved health (76) and are suggested as follows: (i) the microorganism can be found in high abundance in healthy organisms and decreased abundance in the ones suffering from a disease; (ii)

the microorganism can be isolated from a healthy organism and grown in pure culture; (iii) according to the definition of probiotics, the cultured organism should promote health when introduced into a diseased organism; and (iv) because probiotics are by definition administered as live microorganisms, it should be possible to reisolate these microorganisms from the healthy experimental host and identify them as being identical to the original specific causative agent. According to the research about the development of the healthy URT microbiome summarized above (15, 41, 42, 59), *Dolosigranulum* is currently a prime candidate as a next-generation probiotic. However, according to the definition of a probiotic and the translated "probiotic postulates," further exploration of the beneficial functional potential of specific strains of this underexplored lactic acid bacterium is needed before they can be defined as probiotics.

INFECTION MECHANISMS OF THE MAIN BACTERIAL OM PATHOGENS

Since both culture-dependent and culture-independent studies as reviewed above highlight *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* as key otitis media pathogens (77–80), we review their main pathogenesis mechanisms for host respiratory colonization and disease. These virulence mechanisms can be divided into three partially overlapping disease mechanisms: interactions with the nasopharyngeal epithelium, interactions with the host immune system, and formation of polymicrobial biofilms. Of note, since pathogenicity is strain specific, virulence factors can vary between distinct strains, which results in different grades of pathogenicity. However, molecular insights into virulence mechanisms will help in the study of probiotic mechanisms of interventions that could prevent or inhibit these key pathogenic steps as new alternative treatment strategies for OM. Probiotics, being living microorganisms expressing a multitude of effector molecules, use multifactorial mechanisms of actions that can all possibly target the virulence mechanisms of the pathogens, such as colonization, toxin production, inflammation, and biofilm formation. In the next paragraphs, the most commonly occurring virulence factors in the three main OM pathogens are discussed.

Interactions with Nasopharyngeal Epithelium

Impact on mucin and toxin production. Before gaining access to the receptors of the epithelial cells, pathogenic invading bacteria must traverse the mucus layer of the nasopharynx. This layer consists of a mixture of water, ions, glycoproteins, proteins, and lipids and serves as an important defense mechanism of the host against invading pathogens (81). Moreover, the epithelial barrier is also important to keep a beneficial symbiosis in the host-microbiota relationship (1). The glycoproteins (with 70% to 80% O-linked glycosylation) in the mucus, also called mucins, are secreted by goblet cells. Although in healthy conditions the mucins help to protect the host mucosae, in diseased conditions like OM, the mucociliary clearance becomes ineffective and an excessive production of mucins will occur (82). Pathogens have developed multiple ways to overcome this mucus layer and get access to the epithelial cells more easily. *S. pneumoniae*, for instance, uses its neuraminidases (NanA and NanB) to cleave the layer and is helped by its capsule to prevent entrapment in the mucus (Fig. 4) (77, 83). However, at least 98 different capsule serotypes are known to date, while only a limited number of these serotypes are associated with colonization and disease (84). Protein D, on the other hand, is an outer membrane protein, present on the surface of all *H. influenzae* strains, which causes dysfunction of the nasopharyngeal cilia (85).

In humans, more than 20 mucin genes have been identified (86). Upregulation of *MUC5B*, *MUC5AC*, and/or *MUC4* is especially linked with OM (71, 86). Of note, the main pathogens that are involved in OM can upregulate *MUC5AC* (87–90). Furthermore, in a culture model of human middle ear epithelium, whole-cell lysates of the three pathogens induced upregulation of *MUC2*, *MUC5AC*, and *MUC5B*. (91). In mice, *MUC5B* appeared to be required for mucociliary clearance, for controlling infections in the airways and middle ear, and for maintaining immune homeostasis in mouse lungs, whereas *MUC5AC* was dispensable (92).

Pathogens can also attack nasopharyngeal epithelial cells by production of toxins.

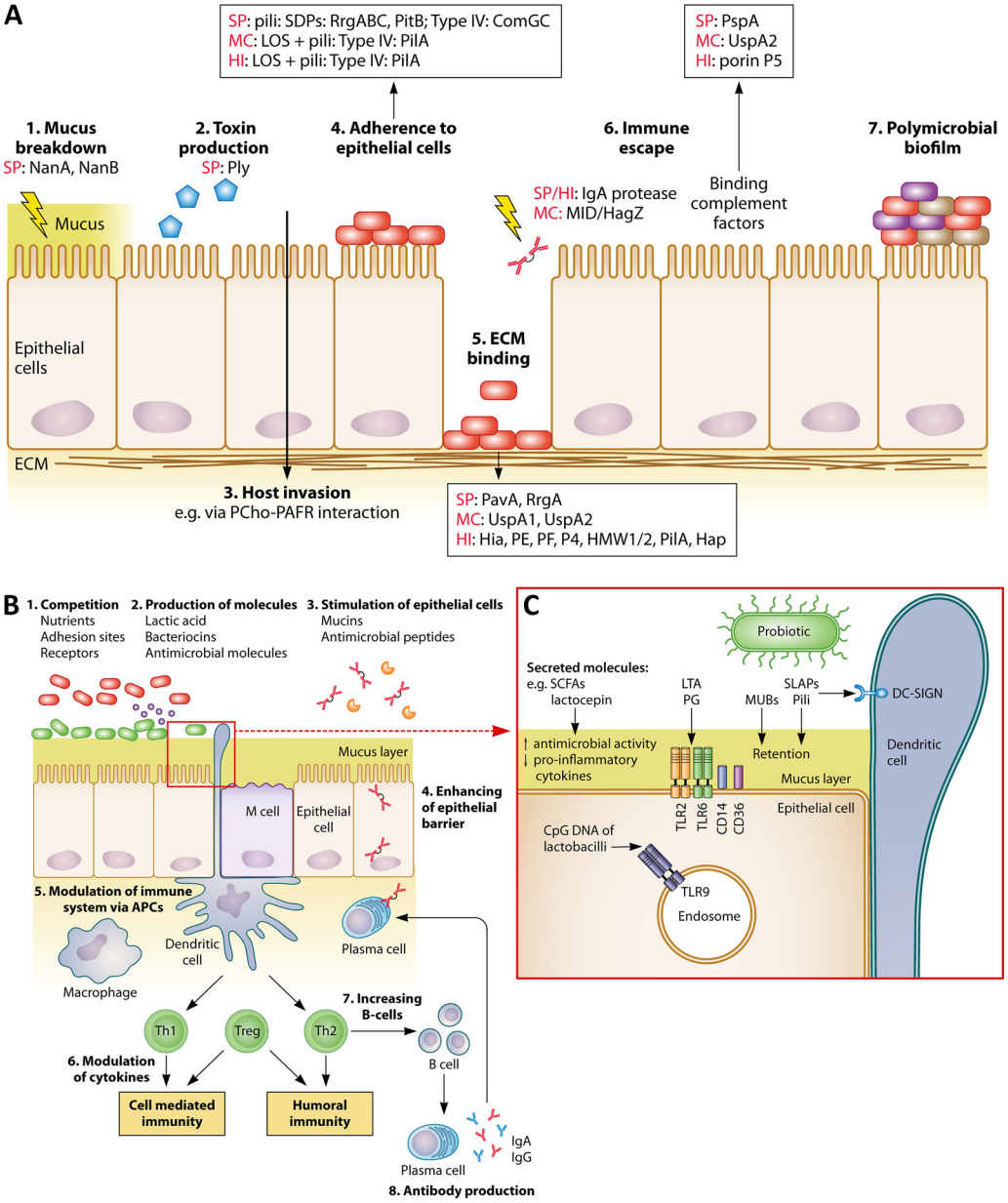

FIG 4 Comparison between pathogenic and probiotic interactions with the nasopharyngeal epithelium and immune system. (A) Pathogens can interact with nasopharyngeal epithelium and host immune system via (1) breakdown of mucus; (2) production of toxins; (3) invasion of the host; (4) adhesion to the epithelium; (5) binding of ECM via microbial surface components recognizing adhesive matrix molecules (MSCRAMM); (6) escaping immune responses; and (7) the formation of a polymicrobial biofilm. Different frequently occurring pathogenic effector molecules are specified for each (A)OM pathogen. SP, *S. pneumoniae*; MC, *M. catarrhalis*; HI, *H. influenzae*; ECM, extracellular matrix; NanA/B, neuraminidases; Ply, pneumolysin; PCho, phosphorylcholine; PAFR, platelet-activating factor receptor; PavA, pneumococcal adhesion and virulence A; UspA, ubiquitous surface protein; Hia, *H. influenzae* adhesion; HMW1/2, high-molecular-weight molecules 1/2; Hap, *Haemophilus* adhesion protein; PspA, pneumococcal surface protein A. It should be noted that not all pathogenic strains or serotypes carry these effector molecules. (B) Postulated beneficial modes of action of URT probiotics. In agreement with beneficial activities in the gut, probiotics could also perform such activities in the URT by, for instance, (1) competition with pathogens for nutrients, adhesion sites, and receptors; (2) production of antimicrobial molecules, such as bacteriocins and lactic acid; (3) stimulation of epithelial cells to modulate mucin and antimicrobial peptide production; (4) enhancement of the epithelial barrier; (5) modulation of the immune system via APCs; (6) modulation of cytokine production; (7) stimulation of increased B-cell production; and (8) stimulation of antibody production. (C) Interaction of several probiotic effector molecules with their receptors localized on the epithelial/dendritic cells or endosomes. APC, antigen-presenting cells; LTA, lipoteichoic acid; MUB, mucus binding protein; PG, peptidoglycan; SCFA, short-chain fatty acids; SLAP, surface-layer-associated protein; Th1/2, T helper 1/2 cells; Treg, regulatory T cells. The figure is based on data described herein in "Infection Mechanisms of the Main Bacterial OM Pathogens" and obtained from references 174, 185, and 281.

Current knowledge indicates that, of the three main OM pathogens, only *S. pneumoniae* produces such an exotoxin. Pneumolysin binds to cholesterol in cell membranes, forming oligomers and creating transmembrane pores (93). It is produced by almost all pneumococcal isolates and can decrease mucosal clearance in the upper airways (94–96). *Haemophilus* and *Moraxella* are Gram-negative bacteria that have lipooligosaccharides (LOS), or endotoxins, in their cell wall (79). Both *M. catarrhalis* and *H. influenzae* use them for adhesion, biofilm formation, and resistance to complement killing (79, 97–101). Not surprisingly, the presence of LOS is an important trigger for OM development in chinchilla models (98, 100).

Adhesion to epithelial cells and extracellular matrix (ECM). As for most mucosal pathogens, adhesion to the nasopharyngeal epithelium is thought to be another key step in pathogenesis. Pili are long and thin proteinaceous protrusions of the cell surface present on specific Gram-positive and Gram-negative bacteria. Their molecular structure can be very diverse. Two types of sortase-dependent pili have been reported in *S. pneumoniae* (102–104). Type 1 pili are thermosensitive, as they are not induced in environments where the temperature is lower than 31°C (105), which suggests that the pathogen uses different virulence mechanisms in cooler anatomic sites, such as the nares, than in warmer sites, such as the nasopharynx/lungs. Both type 1 and type 2 pili have been shown to play an important role in adherence of *S. pneumoniae* to epithelial cells, although the corresponding host receptors are as yet unidentified (102, 104). Furthermore, *S. pneumoniae* uses the sortase-independent type IV pili for binding and internalization of exogenous DNA, which can lead to incorporation of new genetic material and resistance to antibiotics and vaccines (106). This type IV pilus is only assembled during bacterial competence (107), but its role in adhesion is unknown.

Type IV pili are also quite common in Gram-negative pathogens, with *M. catarrhalis* and *H. influenzae* expressing them as well to use for adhesion (108–111). Although the exact mechanism of adhesion is not yet unraveled, the pili of *H. influenzae* have been shown to bind the intercellular adhesion molecule 1 (ICAM-1) receptor (110), which is also used by other OM pathogens, such as the rhinovirus (112).

Adhesion to epithelial cells is also facilitated by the *Haemophilus* adhesion protein (Hap) (80, 113). Furthermore, β-glucan receptors on the surface of monocytic cells and macrophages are involved in the adherence and nonopsonic entry of NTHi, which does not express capsular polysaccharides (114). Moreover, phosphorylcholine (PCho) can be covalently attached through its phosphate group to the LOS of *H. influenzae* (80), similar to the way PCho can be bound to lipoteichoic acid (LTA) of *S. pneumoniae* (115). PCho enhances the bacterium's survival in the respiratory tract, as it increases adherence and invasion (116–119).

Underneath the epithelial cells, the extracellular matrix (ECM) of the host appears to be a major target for colonization by the key OM pathogens, because they all contain microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Fig. 4) (120–126). These molecules bind fibronectin, fibrinogen, laminin, and/or collagen I and, thus, have an important function in host invasion.

Interactions with Host Immune System

Proinflammatory interactions in the host. Once the OM pathogens have invaded and crossed the epithelial barrier, they interact with antigen-presenting cells (APCs) and stimulate them to secrete different cytokines, which play a pivotal role in the inflammatory responses. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), for example, have been thought to initiate the acute inflammatory response in OM (127). Moreover, in a chinchilla model, both IL-1β and TNF-α appear to regulate mucin production in a dose- and time-dependent way, especially the *MUC5AC* gene (128). IL-8, on the other hand, is an important attractant for neutrophils (129). Si et al. (2014) (130) observed increased mRNA levels of interferon gamma (IFN-γ), TNF-α, IL-1β, and IL-6, while protein analysis via enzyme-linked immunosorbent assay (ELISA) only recorded higher TNF-α and IL-1β concentrations in MEF samples of OME children compared to those of non-OME children. Similarly, ELISA of MEF samples of OME children showed a

positive correlation between the concentrations of the proinflammatory cytokines IL-1 β, IL-6, IL-8, and TNF- α and the amounts of OM pathogens in the MEF (131).

Immune responses can be activated by specific pattern recognition receptors (PRRs), often Toll-like receptors (TLRs), which are found on epithelial cells, mast cells, dendritic cells, and other APCs. These receptors are trained to trigger host immune responses to bacterial ligands. In the middle ear mucosa of both OM and non-OM patients, TLR2, TLR4, TLR5, TLR6, and TLR9 are found at the mRNA and protein levels, but the correlation between expression levels and OM phenotype differs in different studies (130, 132). Interestingly, the outer membrane protein ubiquitous surface protein A1 (UspA1) of *M. catarrhalis* is able to inhibit the TLR2/NF-κB proinflammatory responses in the host (133). On the other hand, in *H. influenzae*-associated infections, the TLR2-interacting lipoproteins seem to be major triggers of the immune system (80). Moreover, both LTA of *S. pneumoniae* and PCho of *S. pneumoniae* and *H. influenzae* can also induce inflammation in the host via a TLR2-independent mechanism (77, 134, 135).

As already mentioned, pneumolysin is a very important virulence factor of most serotypes of *S. pneumoniae*, but it has also an effect on the host immune response. It can activate $CD4^+$ T-cells by impairing the respiratory burst of phagocytic cells, by inducing production of chemokines and cytokines, by stimulating complement fixation, and by activating inflammation (77, 94, 136). However, some strains and serotypes have evolved mechanisms to evade the immune responses of the inflammasome (137).

Immune escape factors. To protect themselves against the host's adaptive immune defense, many pathogens directly target antimicrobial molecules or antibodies from the host. For instance, IgA1 proteases produced by *S. pneumoniae* and *H. influenzae* cleave human secretory antibodies like sIgA (Fig. 4) (78, 138), and the *M. catarrhalis* immunoglobulin D (IgD) binding protein/hemagglutinin (MID/Hag) binds soluble IgD (139). Furthermore, PCho protects *H. influenzae* against IgG binding and the human antimicrobial cathelicidin LL-37 (140, 141). In addition, extracellular DNA (eDNA) of *H. influenzae* can neutralize human β-defensin (HBD) (141–145), while the pneumococcal surface protein A (PspA) of pneumococci can bind the antibacterial lactoferrin (146). Furthermore, a camouflage strategy to protect against antibody recognition is reported to be used by the variable LOS of *H. influenzae* (147) and the orientation-switching lipoprotein P6 of *S. pneumoniae* (148). Pathogens can also evade the host's immune system by, for instance, binding complement factors. The pneumococcal surface protein C (PspC or CbpA), UspA2 of *M. catarrhalis*, and the porin P5 of *H. influenzae* both prevent complement-mediated opsonization (80, 146, 149–154).

Polymicrobial Biofilm Formation

A pathogenesis mechanism that receives a lot of attention in COM and OME is mono- and polymicrobial biofilm formation by OM bacterial pathogens (Fig. 4) (110, 155–164). These studies indicate that the presence of biofilms causes OM episodes to recur more often. By investigating middle ear mucosa biopsy specimens of OME children with confocal scanning laser microscopy and fluorescence *in situ* hybridization (FISH), Hall-Stoodley et al. (155) observed the presence of all three main OM pathogens in the biofilms. *M. catarrhalis*, however, seemed to be present in polymicrobial infections more often than in monomicrobial infections (165). These observations suggest that other bacterial pathogens can facilitate persistence of and/or infection by *M. catarrhalis*. Indeed, although bacteria often compete with each other for, e.g., nutrients and receptors, in many cases they collaborate for the greater common good. The formation of an extensive exopolysaccharide or exopolymeric substance (EPS) matrix, for example, results in general protection of the inhabitants of the biofilm. Additionally, in a polymicrobial biofilm, β-lactam-resistant *H. influenzae* and *M. catarrhalis* can protect *S. pneumoniae* against β-lactam antibiotics, while *S. pneumoniae*, on its turn, protects the other two pathogens against macrolide killing (166, 167). Furthermore, Cope et al. (168) observed upregulation of type IV pili of *H. influenzae* and increased H_2O_2 production by *S. pneumoniae* when they were growing together in a biofilm. The

exact functions of these molecules in a polymicrobial biofilm are, however, not yet clear.

POTENTIAL OF PROBIOTICS AGAINST OM AND THEIR MOLECULAR MECHANISMS

Among the bacteria that are more prevalent in healthy subjects than in OM patients are potential probiotics that can contribute to better ear and upper respiratory tract health. However, as we suggested by introducing the translated "probiotic postulates," not only is higher abundance in healthy persons compared to diseased persons important, but also, a causative relationship with health-promoting effects should be demonstrated before a strain can be designated probiotic. Thus, for a microbial strain to be probiotic, its health benefits should first be shown in relevant *in vitro* and *in vivo* model systems and then ultimately be documented in clinical trials that can substantiate causal health relations for the specific probiotic applied. Of note, as mentioned above, LAB, which are widely applied as gastrointestinal probiotics, are also among the interesting probiotic candidates for the URT based on several NGS studies mentioned above (and summarized in Table 2); therefore, various examples will be given for this group of bacteria.

Possible Application Routes and Formulations for URT Probiotics

Although the URT mucosa is the target site and most health-promoting mechanisms of action of probiotics happen at that location, most of the human studies conducted are performed with orally administered probiotic LAB. Orally applied probiotics could benefit the URT via systemic immune effects, but it is also possible that orally ingested probiotics transfer to regions of the URT via the nasopharynx, since all these human body sites and their associated microbial niches are interlinked (1). *Lactobacillus rhamnosus* GG, for example, has also been shown to colonize the tonsils when administered in a dairy formulation containing 10^{10} CFU daily for 3 weeks (169). In addition, *L. rhamnosus* GG was recovered from adenoids (100% recovery by quantitative PCR [qPCR]) and middle ear fluid (MEF) (21% recovery by qPCR) after oral consumption in a dairy formulation for 3 weeks at ca. 1.6×10^{10} CFU/dose (170, 171).

Nasal applications on the other hand, have the advantage of promoting a more direct contact of the applied probiotics with the nasopharyngeal niche and pathogens. Furthermore, by using this delivery route, bacteria do not have to survive the stressful transit through the gastrointestinal tract for the systemic immune stimulation. In addition, the oronasopharyngeal cavity is more accessible and generally populated by a less complex and less dense microbiota than the gut, which makes nasal delivery an interesting alternative to the classical oral route. However, several other barriers emerge to which probiotics should be properly adapted, as further discussed below.

Both oral and nasal administration generally need a drying step in the formulation of the product in order to properly store the probiotics and increase shelf life, but drying can reduce the activity of the probiotic bacteria. Sufficient viability of the strains and preservation of their morphological and metabolic properties after drying are indispensable for probiotics, and consequently, specific pharmaceutical biotechnological strategies are needed. Several protective approaches, such as the addition of protective agents, accurate control of the drying process parameters, and prestressing the probiotics prior to drying, can be used to enhance the viability of strains (172). In addition, safeguarding the presence of cell surface molecules, such as pili, is crucial, as these molecules can be important for adherence to respiratory cells and immunological stimulation (173). However, in many cases, these specific characteristics of the probiotic products administered in clinical studies are unknown.

Clinical Studies with Topical Application of Probiotics

Currently, only a limited number of clinical trials have been performed with potential probiotics in relation to health benefits to the URT of the host (Table 3). Furthermore, the current data on the clinical efficiency of probiotics for OM are not univocal.

TABLE 3 Overview of some of the most pioneering clinical trials where the probiotic strains are applied directly to the URT

URT disease	Probiotic strain(s)	Vehicle	No. of subjects	Dose and duration	Results[a]	Reference
OM	*S. mitis*, *S. sanguis*, *S. oralis*	Saline spray	108	5×10^8 CFU/ml in 150-μl saline suspension per nostril 2×/day intranasally, 10 days/mo for 2 consecutive mos	Cured (↑), 42% in streptococcal group vs. 22% in placebo group ($P = 0.02$); recurrence of OM (↓), 40% vs. 51% ($P = 0.04$); nasopharyngeal microbiota change, ns	176
OM	*S. mitis*, *S. sanguis*, *S. oralis*	Saline spray	43	10^7 CFU/ml in 50-μl saline suspension per nostril 1×/day intranasally for 4 mos	Episodes of OM, 44% in streptococcal group vs. 40% in placebo group (ns); nasopharyngeal microbiota change, ns	177
OM	*S. sanguinis* NCIMB 40104, *L. rhamnosus* NCIMB40564	Saline spray	60	5×10^9 CFU/ml in 100-μl saline suspension per nostril 2×/day for 10 days	Recovery, 7/19 patients in *S. sanguinis* group vs. 1/17 in placebo group ($P < 0.05$) and 3/18 patients in *L. rhamnosus* group ($P = 0.60$ compared with placebo group); nasopharyngeal microbiota change, ns	180
OM	*S. salivarius* 24SMB	Saline spray	100	10^{11} CFU/ml in 50-μl saline suspension per nostril 2×/day intranasally 5 days/mo for 3 consecutive mos	Recurrence of OM (↓), children without OM, 30% in streptococcal group vs. 14.9% in placebo group ($P = 0.076$); antibiotic use (↓), 70% versus 83.0% ($P = 0.13$); recurrence of OM, 13.6% after colonization of *S. salivarius* 24 SMB vs. 42.8% without colonization	181
OM	*S. salivarius* 24SMB, *Streptococcus oralis* 89a	Saline spray	267	10^9 CFU/dose, 2×/day intranasally, 1 wk/mo for 3 consecutive mos	Recurrence of OM (↓), 9.4% of children had the same number of AOM episodes in spray-treated group versus 68.5% in control group	179
General URT inflammation	Mixture of 9 *Lactobacillus* spp. and 4 *Bifidobacterium* spp.	Honey and pollen in water spray	22	10^{11} CFU/ml in 200-μl suspension per nostril intranasally as 1 dose	No untoward effects; no significant difference in SNOT-22 scores obtained after challenge with LAB and sham treatment; nasopharyngeal microbiota change, ns	182

[a] ↑, increase in active group; ↓, decrease in active group; nasopharyngeal microbiota change, difference in distribution of bacteria between active and placebo groups observed via culture-dependent techniques and/or PCR; ns, not significant.

Both oral and topical intake of probiotics has been explored in recent years (as reviewed in Marom et al. and Niittynen et al. [174, 175]). The oral administration route especially aims at enhancing immune responses systemically (mainly via the gastrointestinal immune cells). On the other hand, topical application of the probiotic strains directly in the URT, e.g., via a nasal spray, might be a better administration route to directly target the OM pathogens, but this has only been explored for a limited number of probiotic species so far. Some of the best documented LAB probiotics for topical application are alpha-hemolytic *Streptococcus* (AHS) bacteria (174). A combination of two strains of *Streptococcus mitis* and *Streptococcus sanguis* and one strain of *Streptococcus oralis*, all isolated from the eustachian tube opening of healthy children and able to inhibit growth of *S. pneumoniae*, was used in two Swedish studies. In the first one, 108 otitis-prone children were investigated after daily nasal administration of the AHS mixture (7.5×10^7 CFU per intake) or placebo for 10 consecutive days. The AHS treatment group experienced fewer recurrences of AOM than the placebo group as monitored for a 3-month period (176). However, the second study, which tested the same mixture of AHS (5×10^5 CFU per intake) in 43 children with recurrent OM for 4 months, did not see a difference in AOM recurrences and did not detect significant changes in the nasopharyngeal colonization of the children (177). This difference could be due to the smaller amount of streptococci administered in the latter study (Table 3). In addition, after their colonization, safety, and tolerability were investigated (178), a mixture of two other *Streptococcus* strains, *Streptococcus salivarius* 24SMB and *S. oralis* 89a, was tested in an Italian cohort of 267 children (179). A reduction in the reoccurrence of AOM was observed in all children using the spray, while only 50% of the children in the control group experienced fewer AOM episodes. Skovbjerg et al. (180) used lactobacilli in a similar study. They compared the administration of *S. sanguinis* NCIMB 40104, *L. rhamnosus* NCIMB 40564, or a placebo in 60 children with serous OM. In both treatment groups, ca. 50% of the children showed improvements or were cured (9/19 in the *Streptococcus* group and 9/18 in the *Lactobacillus* group), while this number decreased to only 18% (3/17) in the placebo group. The spray treatment did not alter the composition of the nasopharyngeal microbiota (although it was only monitored with culture techniques) or the cytokine patterns (IL-1β, IL-6, IL-8, and IL-10) in the middle ear fluid (180). More recently, *S. salivarius* 24SMB also showed promising results (181). Children who administered the strain in each nostril twice per day for 5 consecutive days during 3 consecutive months showed fewer episodes of AOM and received less antibiotics over a 6-month period. In addition, Mårtensson et al. (182) reported the successful nasal administration to healthy adults of promising *Lactobacillus* and *Bifidobacterium* strains, isolated from honeybees and proven to have antimicrobial activity against the important human URT pathogens *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The spray did not increase URT inflammation as tested with a cytokine microarray representing 30 cytokines/chemokines and mediators involved in type 1 and 2 inflammatory responses. Moreover, no adverse effects were observed after administration. Since ancient times, honey has been used to treat respiratory diseases and its medicinal properties have received considerable recognition in medicine (183). However, whether the gut lactobacilli of the honeybee are partially responsible for the antimicrobial and healing activities of honey remains to be substantiated.

As summarized above and in Table 3, although various clinical benefits have been reported, the randomized-controlled studies with probiotics do not all show efficacy. This could be explained by the fact that the probiotic strain applied was not optimally selected or, perhaps, administered (e.g., too low a dose or too short a duration) for the URT condition targeted or because most of the study participants (hosts) were not responsive to the selected probiotics. Detection methods, host genetics, too severe inflammation, or too severe microbiome dysbiosis could indeed influence (measured) responses to probiotic treatment, as also shown for gastrointestinal applications of probiotics, highlighting the need for patient stratification (e.g., see Claes et al. [184]). Therefore, it can be anticipated that knowledge about the molecular mechanisms of

TABLE 4 Overview of some of the most important molecular mechanisms of probiotics

Type of action	Mode of action	Molecule(s)/structure(s)/adjustment	Example(s)	Reference(s)
Competition	Adhesion sites and receptors	Pili or fimbriae	SpaCBA pili in *L. rhamnosus* GG	173, 187
		Lectins	Llp-1 in *L. rhamnosus* GR-1	190
	Nutrients	General better adaptation		
	Environment	General better adaptation		
Production of molecules	Acids	Lactic acid	General in LAB	197, 200–202
	Proteins	Bacteriocins	Lactacin in *L. acidophilus*, plantacin in *L. plantarum*, nisin in *L. lactis*	209, 210, 212
	Others	H_2O_2	In many LAB	216
		Quorum-sensing interaction	AI-2 in many LAB	219 (for *L. rhamnosus* GG)
Stimulation of epithelial cells	Mucins	Unknown	*L. rhamnosus* GG	197
	Antimicrobial peptides (defensins, lysozymes, cathelicidins, etc.)	Muramyl dipeptide motif	M-tri-Lys in *L. salivarius*	240
Enhancement of epithelial barrier	Upregulation of tight junctions	Secreted soluble proteins	Msp1/p75 and Msp2/p40 in *L. rhamnosus* GG	231
		Unknown	*L. plantarum* WCFS1	230
	Barrier repair	Secreted soluble proteins	Msp1/p75 and Msp2/p40 in *L. rhamnosus* GG	232, 233
Modulation of immune system (via APCs, modulation of cytokines, increased B cells, antibody production)	MAMPs	Surface layer-associated proteins (SLAPs)	SlpA in *L. acidophilus* NCFM	248
		Pili	SpaCBA pili in *L. rhamnosus* GG	254, 255
		EPS	*L. casei* Shirota, *L. plantarum*	261, 262
		LTA	In several LAB	257–260
		CpG-rich DNA	In several LAB	263
	Secreted effector molecules	Proteases	PrtP protease or lactocepin in *L. paracasei*	264
		Short-chain fatty acids (SCFAs)	In several LAB (direct or indirect through cross-feeding)	266–269

action of the probiotics in the URT, and better molecular knowledge of OM pathogenesis, will facilitate the selection of the most optimal probiotic strain for each condition and the subjects benefiting most from their application (as potential responders). In the next paragraphs, potential probiotic mechanisms of action against infection by OM pathogens are discussed. Although little is yet documented about the potential protective characteristics of nasopharyngeal probiotics, we have rationalized these mechanisms similarly as for the gastrointestinal tract (GIT) (185).

Properties That Can Be Rationalized To Be Important for URT Probiotics

Since most clinical studies with URT probiotics performed so far have been done with lactic acid bacteria (LAB), and since current microbiome studies also suggest a potential role for LAB (Table 2), potential mechanisms of action of probiotics will be explored here mainly for LAB (summarized in Table 4). Moreover, LAB have an advantage over other, less well studied health-related taxa, such as *Corynebacterium* (Table 2), because they have a long history of safe use (generally recognized as safe [GRAS] and qualified presumption of safety [QPS] status) in fermented foods, which is important for future applications.

Adaptation Mechanisms Rationalized for URT Probiotics

Considering the fact that most URT pathogens adhere strongly to the nasopharyngeal or middle ear epithelium, at least temporarily, during their infection process (as discussed in earlier paragraphs), it is reasonable to envisage that probiotics to be applied in the URT should be able to persist temporarily at the mucosa to compete with these pathogens, especially considering that nasal clearance is less than 20 min (186).

Selecting highly adherent probiotic strains is generally part of the screening platforms, although there is no consensus in the literature that gastrointestinal probiotics should be able to strongly adhere to the mucosa. Successful gastrointestinal probiotics, such as *Lactobacillus rhamnosus* GG, show a high capacity to adhere to human intestinal epithelial cells and mucus due to the presence of adhesive heteromeric SpaCBA pili (173, 187). More specifically, the tip pilin SpaC acts as a mucus binding protein (MUB). Whether the SpaCBA pili are also important for adherence to respiratory and nasopharyngeal epithelium cells is not known at present. In addition to pili, other sortase-dependent proteins (SDPs) could promote adherence of lactobacilli, as well as related potential probiotics, to the respiratory tract epithelium (188). For instance, we recently found indications for a novel type of sortase-dependent pili or fimbriae in the nasopharyngeal *Lactobacillus casei* AMBR2 strain (189). Other surface proteins that are linked to adherence to the host epithelium are lectins, i.e., proteins that bind carbohydrates with high specificity. For instance, the lectin-like protein 1 (Llp-1) of *L. rhamnosus* GR-1 has been shown to play a tissue-specific role in adhesion to vaginal epithelium (190) but not gastrointestinal and endocervical cells, suggesting that lectins could also mediate tissue-specific adhesion to the URT niche.

Being able to strongly adhere to the nasopharyngeal or middle ear epithelium will probably not be sufficient to efficiently compete with the OM pathogens and to sufficiently interact with the human host cells to confer beneficial effects. It can be hypothesized that the applied probiotics should also be able to adapt to the specific host nutritional environment and stress conditions of the URT. Indeed, the conditions in the gut and the URT are not comparable, as they differ substantially in oxygen level, pH, relative humidity, travel distance and time, temperature, etc. (1). The thickness of the EPS layer of *L. rhamnosus* GG, for example, has been shown *in vitro* to increase in a neutral pH (cf. URT) compared to its thickness in an acidic environment (cf. gut), which causes pili of *L. rhamnosus* GG to unfold and be more accessible for interaction with proteins (191), but whether this is also true *in vivo* remains to be substantiated. Further mechanistic studies are certainly needed to define the most important characteristics of candidate probiotic bacteria in the URT. At present, a standard model is lacking for *in vitro* URT adhesion assays (192), but several cell lines are used, such as A549 lung epithelial cells (193), Calu-3 human bronchial cells (194), FaDu hypopharyngeal cells (195), Detroit 562 pharyngeal cells (124), and CCl-23 laryngeal cells (196). In contrast to the interaction with the gut epithelium, mucosal adhesion of lactobacilli to the nasopharyngeal epithelium has not been extensively studied. However, by *in vitro* assays, Guglielmetti et al. (195) observed that *Lactobacillus helveticus* MIMLh5 was able to adhere to FaDu hypopharyngeal carcinoma cells and antagonize the typical sore-throat pathogen *S. pyogenes*. The model gastrointestinal probiotic *L. rhamnosus* GG has also been shown to inhibit the adherence of *S. pneumoniae* to the laryngeal cell line CCL-23 in a time- and dose-dependent way (196) and to significantly decrease the adhesion of *M. catarrhalis* to Calu-3 human bronchial cells (197).

The reduced pH stress (pH 6.3 and 7 in nasal cavity and nasopharynx, respectively), lower temperature, and higher oxygen level (1) in the URT compared to those in the GIT can be hypothesized to favor other probiotics than the classical GIT ones. At present, the available nutrients and other stress factors in the URT are not well characterized, but it can be rationalized that the probiotics will have to adapt to low concentrations of free carbohydrates and iron (198), as well as to the presence of antimicrobial molecules in the mucus, such as lysozyme, lactoferrin, and PLUNC (palate, lung, and nasal epithelial clone) proteins (199). For instance, our recently isolated *L. casei* AMBR2

strain from the nasopharynx is catalase positive (while most other *Lactobacillus* species are catalase negative), suggesting a role for catalase in adaptation to the oxidative environment of the URT (189). Indeed, URT lactobacilli will have to withstand other stresses than in the GIT: they will not necessarily have to resist gastric digestive enzymes and bile acid stress, unless immunomodulatory effects are also aimed for via the gastrointestinal immune system.

Probiotic Mechanisms Rationalized for URT Probiotics

Direct antimicrobial actions against OM pathogens. In addition to competition for adhesion sites, probiotics can directly inhibit pathogens by producing antimicrobial molecules, such as lactic and acetic acid, bacteriocins, and hydrogen peroxide, in their microenvironment (Table 4) (185). These molecules can inhibit both Gram-positive and Gram-negative bacteria, but of course, the most active mechanism will depend on the exact pathogen(s) that are targeted by probiotic application. Organic acids like lactic and acetic acid can mainly be inhibitory against Gram-negative bacteria, since their undissociated form can enter the bacterial cell and dissociate in the cytoplasm (200–202). In 2006, lactic acid was documented to be the active antimicrobial molecule of lactobacilli against *Salmonella enterica* serovar Typhimurium (201, 203, 204). However, lactic acid has also been shown to permeabilize the Gram-negative outer membrane of pathogens like *Escherichia coli* O157:H7, *P. aeruginosa*, and *S.* Typhimurium by utilizing a fluorescent-probe uptake assay and sensitization to bacteriolysis (200). Furthermore, in spent culture supernatant of lactobacilli, lactic acid was shown to play a crucial role in the antibacterial activity against *M. catarrhalis* (197). This makes it a promising molecule to inhibit Gram-negative URT pathogens like *M. catarrhalis* and *H. influenzae*. However, lactic acid is not the only active molecule which can be produced by lactobacilli (205). Species- and strain-specific bacteriocins are produced by many lactobacilli: several *Lactobacillus acidophilus* strains, for example, produce lactacin (206–208), and many *Lactobacillus plantarum* strains produce plantaricin (209, 210). By the formation of pores or inhibition of cell wall synthesis, bacteriocins exert their antimicrobial action against (often closely related) bacteria. In addition, seven heat-stable antibacterial peptides active against the enteroaggregative *E. coli* strain EAEC042, *S.* Typhimurium, and *S. aureus* were isolated from *L. rhamnosus* GG supernatant (211). The genome sequence of *L. rhamnosus* GG revealed bacteriocin-related genes, which suggests possible production of these antimicrobial peptides (187). However, as far as we know, no bacteriocin of lactobacilli has yet been demonstrated to have antimicrobial activity against OM pathogens, although *S. pneumoniae* is sensitive to nisin, a bacteriocin produced by *Lactococcus lactis* (212). Furthermore, lactobacilli like *L. rhamnosus* GG and *L. rhamnosus* GR-1 contain lectin-like proteins which are shown to inhibit and/or structurally disrupt pathogenic biofilms (190, 213), while a dairy drink containing *L. casei* Shirota has been reported to reduce biofilm formation on voice protheses (214). Pericone et al. (215) observed the bactericidal effect of H_2O_2, produced by *S. pneumoniae*, against its coinhabitants of the URT, such as *H. influenzae* and *M. catarrhalis*, suggesting this mechanism of action might also be mediated in the URT; however, little evidence is yet available. In other human body niches, such as in the vagina of healthy women, H_2O_2 production by lactobacilli was also proposed as an important antimicrobial mechanism (216). However, since the molecule is highly unstable, this mechanism is quite controversial (217).

Another way of looking at production of antimicrobial molecules is the production of molecules that interact with cell-cell communication of pathogens. Quorum sensing, a system of stimuli and responses correlated to population density, might modulate pathogen infection success by coupling gene expression of immune-alarming virulence factors only to high densities (218). The *luxS* gene is responsible for the production of autoinducer-2 (AI-2), an important interspecies quorum-sensing molecule, in both Gram-negative and Gram-positive bacteria (218). Most lactobacilli contain this gene and secrete AI-2 (e.g., *L. rhamnosus* GG [219]), as do the OM pathogens *S. pneumoniae* (220) and *H. influenzae* (221). AI-2 is an important factor in biofilm formation by *S. pneu-*

moniae and *H. influenzae* (220, 221). Furthermore, a mutation in the *luxS* gene causes reductions in virulence and persistence in a murine model of nasopharyngeal carriage of *S. pneumoniae*, while a *luxS* mutation increases the virulence of *H. influenzae* in a chinchilla model (222–224). In contrast to these pathogens, *M. catarrhalis* cannot produce AI-2 itself, as it does not contain the *luxS* gene. However, its biofilm formation is promoted by the production of AI-2 by *H. influenzae* (225). Disrupting AI-2 transport, antagonizing its signaling, inhibiting AI-2 production, or quenching AI-2 would thus be possible strategies to interfere with the interspecies communication in OM infections. However, since the AI-2 synthase LuxS also interferes with general cell metabolism (226), the role of quorum sensing in pathogen exclusion is difficult to investigate.

Enhancement of the nasopharyngeal epithelial barrier. Another documented probiotic mechanism for the GIT is enhancement of the epithelial barrier function, as reviewed recently by Bron et al. (Table 4) (227). Although barrier defects are coupled to many URT diseases, such as OM (228), barrier enhancement by probiotics has not yet been explored in detail for the URT niche (229). And yet, it is possible to translate possible mechanisms for barrier enhancement, such as enhancement of tight junction functioning, from the GIT to the URT. In an *in vivo* study, *L. plantarum* WCFS1 was shown to induce changes in the intestinal epithelial tight junctions, which was demonstrated by an increased presence of zonula occludens-1 and occludin, two tight junction proteins (230). In addition, two soluble proteins produced by *L. rhamnosus* GG, Msp1/p75 and Msp2/p40, were demonstrated to protect the tight junctions in Caco-2 cell monolayers from hydrogen peroxide-induced disruption (231). Furthermore, these proteins also prevented TNF-induced apoptosis of epithelial cells in cultured cells and *ex vivo* colon organ culture models (232). In addition to their preventive function, both p75 and p40 have been shown to have potential to repair the intestinal barrier (232, 233), which is of interest for URT therapy (234) but should be further substantiated for nasal and respiratory epithelial cells. Also, symbiont-generated lactate has been shown to support intestinal epithelial cell regeneration (235).

Another epithelial barrier function-promoting mechanism is the induction of antimicrobial peptides like defensins, which protect mucous membranes against invading microorganisms (199, 236). The mechanism of the antimicrobial activity of defensins is multiple: the construction of pores in the membrane of pathogens is the most important one, but they can also inhibit bacterial toxins, such as pneumolysin of *S. pneumoniae* (237). On the other hand, defensins can influence the immune system to produce proinflammatory cytokines and chemokines. Of the human β-defensins (HBDs), HBD-2 is the most potent antimicrobial peptide (238–240). HBD-2-mediated killing of some strains of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* has been reported at low concentrations (241) and can be induced by probiotics (242). In addition to HBD-2, human α-defensins 1 to 4, which are expressed by neutrophil granules, are important in the phagocytosis-mediated killing of bacteria. *H. influenzae* is especially sensitive to this kind of defensins (243).

Furthermore, human epithelial cells can produce other antimicrobial proteins, such as lysozymes, cathelicidins, C-type lectins, and ribonucleases, which often attack cell wall structures and/or the bacterial membrane. Lysozyme degrades the peptidoglycan of the bacterial cell wall and can kill *S. pneumoniae* synergistically with HBD-2 (244). Cathelicidins, such as the above-mentioned LL-37, are cationic antimicrobial peptides that also trigger the host's immune system. In a chinchilla model, a cathelicidin was observed to be able to kill the NTHi strain 86028-NP and *M. catarrhalis* 1857; however, *S. pneumoniae* serotype 14 seemed to be less sensitive (245). *L. rhamnosus* GG can upregulate cathelicidin-related antimicrobial peptides (CRAMPs) in mice (246), but little is known about similar effects in humans. Other examples are the induction of mucus and the induction of cytoprotective molecules (reviewed in Madsen [236]).

Enhancement of the (systemic) immune system. Besides the stimulation of the production of antimicrobial molecules of the host, the application of probiotics can also modulate host immune responses, both innate and adaptive immunity (Table 4) (247). Probiotic bacteria can, for instance, modulate the maturation of dendritic cells (DCs)

toward an anti-inflammatory IL-10 profile. The protein SlpA of *L. acidophilus* NCFM, a surface-layer-associated protein (SLAP), has been shown to fulfill this immunostimulating role through interaction with DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Fig. 4) (248). In addition, the stimulatory role of probiotics on regulatory T-cell activity is well explored and seems an important probiotic mechanism in controlling overt inflammatory conditions, although there exist large strain differences for this capacity (249, 250). In asthmatic mice, oral administration of *L. rhamnosus* GG (10^9 CFU every second day for 8 consecutive weeks) has been shown to suppress allergen-induced proliferative responses associated with an induction of T-regulatory cells in both mesenteric and peribronchial lymph nodes (250). In addition, experiments from our laboratory with nasal administration of *L. rhamnosus* GG to mice resulted in the prevention of allergic asthma. Mice which received the probiotic for 8 days showed a decrease in lung IL-13 and IL-5 levels, together with a decrease in bronchoalveolar lavage eosinophil counts and airway reactivity (251). These results point to the potential of nasally administered probiotics to prevent inflammatory responses in the host.

Furthermore, probiotics can stimulate increased mucosal immunoglobulin A (IgA) levels and allergen-specific B- and T-cell responses, which can especially influence allergic diseases, e.g., in the URT (reviewed in Toh et al. and Martens et al. [229, 252]). Guglielmetti et al. (195) explored the immunomodulatory effect of the probiotic strain *L. helveticus* MIMLh5 in FaDu hypopharyngeal carcinoma cells. *L. helveticus* MIMLh5 could reduce the induction of IL-6, IL-8, and TNF-α, while it enhanced the expression of the heat shock protein coding gene *hsp70* (195). Of note, in mice, intranasal administration of *L. rhamnosus* GG for 3 days increased the cytotoxic activity of pulmonary natural killer (NK) cells after infection with influenza virus H1N1. This probiotic strain was also shown to increase the secretion of IL-1β and TNF-α, which resulted in better survival of the mice after 15 days (253).

The exact molecules by which probiotics can exert these immunomodulatory effects are only fragmentarily known. Moreover, the research into these molecules has primarily focused on the GIT environment. Immune priming molecules include microbe-associated molecular patterns (MAMPs), such as LTA and EPS, that can interact with pattern recognition receptors, such as TLRs (Fig. 4) (247). For example, in intestinal cell and monocytic models, pili of the model probiotic *L. rhamnosus* GG, for which several URT benefits have also been mentioned above (173, 187, 196, 197, 219, 231, 232, 250), were observed to have an anti-inflammatory effect on the cells. Increased exposure to the pili, obtained via the use of an EPS mutant of *L. rhamnosus* GG, decreased the IL-8 mRNA induction by 2 times compared to the level in the wild type (254, 255). Similar observations were recently made with a focus on allergy: nasal administration of *L. rhamnosus* GG in mice decreased the allergy-related inflammation in a more pronounced way than nasal administration of *L. rhamnosus* GR-1 (251). A key difference between these two strains is the absence of SpaCBA pili in *L. rhamnosus* GR-1 (256). These results suggest that proper adhesion of the probiotic bacteria to the epithelial cells plays a pivotal role in their immunomodulatory effect.

Other molecules, such as teichoic acids (TAs) and EPS, also show immunomodulatory characteristics in several cell models: LTAs of several *Lactobacillus* strains can bind TLR2 and activate proinflammatory cytokine release (257–260), while EPS molecules of both *L. casei* Shirota and *L. plantarum* seemed to be more immunosuppressive in the gut (261, 262). TLR9, present on endosomes, can, on the other hand, be triggered by unmethylated cytosine-guanine (CpG)-containing DNA. Such CpG-rich DNA is carried by many *Lactobacillus* species (263) and can thus stimulate Th1 responses, leading to cell-mediated immunity. In addition to MAMPs, the secretion of probiotic effector molecules can influence the host's immune system as well. Both in *in vitro* (cell) models and in mice, the production of the cell envelope-associated protease PrtP, also called lactocepin, by *Lactobacillus paracasei* was shown to selectively degrade proinflammatory cytokines (264). A large genome comparison highlighted the presence of this kind of proteases in many *Lactobacillus* strains, suggesting that it is an overarching feature among several strains (265). Other *Lactobacillus* metabolites produced genus wide,

such as lactic acid and acetic acid, also exhibit immunostimulatory effects. In macrophages and neutrophils, several short-chain fatty acids (SCFAs; i.e., sodium butyrate, sodium phenylbutyrate, sodium phenylacetate, acetate, propionate, and butyrate, which could be directly produced by lactobacilli or result from cross-feeding with other bacteria) were shown to inhibit IL-6 and TNF-α but stimulate IL-10 production (266–269). Both *L. acidophilus* and *Lactobacillus johnsonii* La1, for instance, have been shown to stimulate the production of SCFAs like acetate, butyrate, and/or (iso-)valerate in humans and rats, respectively, after oral administration (270, 271). In addition, lactate is also metabolized to butyrate by lactate-utilizing, butyrate-producing bacteria like *Eubacterium hallii* and *Anaerostipes caccae* under the anaerobic conditions of the gut, where it can have additional beneficial functions (272). However, whether these SCFAs are also produced and have a beneficial function in the more oxygen-rich environment of the URT is at present unknown.

Vaccines such as the PCV variants or the 10-valent pneumococcal *H. influenzae* protein D conjugate vaccine (PHiD-CV10) have already shown a decrease in the incidence of OM (273, 274). For these preventive strategies to reduce OM episodes, probiotic bacteria can also be included as vaccine adjuvants. Up to this point, we have mainly discussed the direct immunomodulating effects of living probiotic bacteria. However, probiotics and their MAMPs are also investigated for their potential to ameliorate humoral responses to vaccines when applied as an adjuvant stimulating PRRs. Several studies have demonstrated, for example, that administration of the model probiotic *L. rhamnosus* GG before and/or after vaccination can increase the specific antibody production in the human host, leading to enhanced protection rates (275–278). Induction of a higher concentration of such pathogen-specific antibodies may help to inhibit the pathogen's adhesion to the host's epithelial cells, as observed for *S. pneumoniae* (279). In particular, the PCV vaccines where capsule polysaccharides of *S. pneumoniae* are used, which are less immunogenic than proteins, could benefit from extra immunostimulation via an adjuvant. However, although the URT mucosal immune system is an interesting route for, e.g., vaccination, modulation of immune responses through microbial/probiotic modulations at the URT (for example via nasal application) is currently underexplored.

CONCLUSION

OM is a leading cause of health care visits in children (15), and thanks to major advances in DNA-based bacterial community analyses, our knowledge about the bacterial composition of this disease is steadily increasing. The Human Microbiome Project has already made major efforts for standardization of microbiome-focused studies so that biologically relevant variation in the microbiome composition can be systematically studied. However, due to the use of different sequencing approaches and biological sample material, it is still difficult to define a healthy core microbiome and compare different studies. More uniform sampling protocols and downstream analyses, including algorithms, are required to compare each step adequately, since small details in sample handling can cause large differences in the outcome and interpretation, as reviewed by Vandeputte et al. (38) for gut samples.

Nevertheless, NGS approaches have now substantiated a key role for the classical OM pathogens *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in samples from diseased children. In addition, other potential pathogens, such as *Turicella* and *Alloiococcus*, are gaining attention (Table 5). However, there is currently little knowledge about their virulence factors and pathogenic impact on the human immune system and barrier function. More knowledge, individually and in biofilms, is thus necessary to target specific causal activities of pathogens in the different disease states.

In addition, several of the NGS studies reviewed above have correlated the presence of certain bacteria in the nasopharynx of infants with a healthier status in later childhood. The lactic acid genus *Dolosigranulum*, for example, is increasingly suggested to be a protective bacterial taxon (41, 59, 280). The underlying mechanisms of the potential protective roles of these bacteria are not yet understood.

TABLE 5 Major conclusions and future research points

Category	Discussion
Major conclusions	Otitis media (OM) is a leading cause of antibiotic prescription in childhood, yet antibiotic use should be limited as much as possible
	The main bacterial pathogens contributing to OM are *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*, but microbiome studies of OM also suggest a pathogenic role for other taxa, such as *Turicella* and *Alloiococcus*
	Microbiome studies in healthy children indicate a protective role against OM for lactic acid bacteria, such as *Dolosigranulum*, *Lactobacillus*, and *Lactococcus*
	Microbiome studies give information about the relative abundances of potential pathogens or protective bacteria, but other criteria need to be considered as well, such as those described in (translated probiotic) Koch's postulates
	Both clinical studies and molecular mechanisms suggest that probiotics have potential to reduce OM episodes and the related antibiotic use
Future research points	Microbiome information at the strain level could give more information on strain-specific virulence or protection capacities of specific bacteria
	Molecular mechanistic studies on the potential of probiotics against OM pathogens and their adaptation to the targeted niches are necessary to select for the most optimal probiotics
	Clinical studies in humans will further substantiate the therapeutic effectiveness of such selected probiotics and their influence on the local microbiome

Furthermore, to substantiate the probiotic potential of specific taxa or strains, the importance of validation experiments in which cause-and-effect relationships between the bacteria administered and improved biomarkers for health are demonstrated (see translated "probiotic postulates") cannot be underestimated. Moreover, targeting the microbiome with health-promoting bacteria, such as probiotics, will gain more interest in the future, as the awareness of the negative consequences of antibiotics is rising. Promising clinical studies with several LAB taxa and strains highlight the potential of probiotic bacteria to reduce the disease burden of OM (Table 5). Further clinical substantiation and strain selection of the most optimal health-promoting bacteria against OM will benefit from more molecular insights into both pathogenic and probiotic mechanisms of action, as exemplified above.

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