

Composition and immunological significance of the upper respiratory tract microbiota

Louis Patrick Schenck^{1,2}, Michael G. Surette^{1,2,3} and Dawn M. E. Bowdish^{2,4}

1 Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Canada

2 Michael G. DeGrootte Institute for Infectious Disease Research, McMaster University, Hamilton, Canada

3 Department of Medicine, McMaster University, Hamilton, Canada

4 Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Canada

Correspondence

D. M. E. Bowdish, MG DeGrootte Institute for Infectious Disease Research, McMaster University, MDCL 4020, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada
Fax: +1 (905) 522-6750
Tel: +1 (905) 525-9140 Ext. 21905
E-mail: bowdish@mcmaster.ca

(Received 16 August 2016, revised 30 September 2016, accepted 7 October 2016, available online 1 November 2016)

doi:10.1002/1873-3468.12455

Edited by Wilhelm Just

The intestinal microbiota is essential for nutrient acquisition, immune development, and exclusion of invading pathogens. The upper respiratory tract (URT) microbiota is less well studied and does not appear to abide by many of the paradigms of the gastrointestinal tract. Decades of carriage studies in children have demonstrated that microbe–microbe competition and collusion occurs in the URT. Whether colonization with common pathogens (e.g., *Staphylococcus aureus* and *Streptococcus pneumoniae*) alters immune development or susceptibility to respiratory conditions is just beginning to be understood. Herein, we discuss the biogeography of the URT microbiota, the succession and evolution of the microbiota through the life course, and discuss the evidence for microbe–microbe interactions in colonization and infection.

Keywords: Bacteria–bacteria interactions; microbiota; upper respiratory tract

The study of microbiota began with the discoveries by Antoine van Leeuwenhoek centuries ago with the identification of different bacteria from dental plaque. Early observations indicated that microbial communities influenced the development of the intestinal tract, including the stimulation of antibody production by potential beneficial bacteria, or probiotics [1]. More recent studies have explored the complex, dynamic interactions and influences by external factors such as diet and environment on the gastrointestinal microbiota and its implications on health [2,3]. Some of the earliest studies also indicated that there were general patterns of succession which influenced how communities form and are altered via invasion of new members or destabilization (e.g., by antibiotics) [4,5]. We now know that the microbiota contributes to nutrient production and acquisition, as well as resistance to colonization by pathogens. Interestingly studies in germ-free mice have

demonstrated that the microbiota is required for normal physiological development of the organs such as the gut, lung, and brain [6]. The gut microbiota drives the development of the mucosal immune system, including the initiation of secretion of immunoglobulins [7], the development of T-cell populations [8,9], and stimulating the production of antimicrobial peptides [10]. The respiratory tract microbiota is less well studied, but it is believed that it also contributes to local immune education and the development of respiratory diseases, including asthma and allergy.

The communities of the upper respiratory tract (URT) microbiota (i.e., sinuses, nares, oropharyngeal) are distinct from one another and have unique patterns of colonization and succession. From early infancy, the URT is colonized by several pathogens, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*. These and other bacteria can also

Abbreviations

COPD, chronic obstructive pulmonary disease; Esp, extracellular serine protease; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; OTUs, operational taxonomic units; TNF, tumor necrosis factor; URT, upper respiratory tract.

be referred to as ‘pathobionts’, which generally colonize the host asymptotically, but have the potential to cause disease if they spread from the site of colonization [11]. In fact, some pathobionts such as the *Streptococcus anginosus* group are believed to be the major cause of complicated lung infections such as pleural empyema [12,13]. The composition of the URT microbiota, including the presence of pathogens, changes rapidly during infancy, the process of which is called ecological succession. Ecological succession describes changes within the microbial community over time, which is influenced by both changing habitats (e.g., the developing human tissue) and exposure to new community members (i.e., colonizing bacteria). The progression of the URT microbiota has been shown to impact airway disease later in childhood [14]. Understanding how microbial succession and colonization increases or reduces susceptibility to respiratory diseases such as asthma and allergy and how it promotes or prevents invasion by pathogens may inform novel interventions. This review describes the current understanding of how the URT microbiota influences health, infection, and inflammation.

Biogeography of the URT microbiota

The major functions of the URT are to heat, humidify, and filter air through several compartments (i.e., nares vestibules, nasal cavity/meatus, nasopharynx, oropharynx, and pharynx) before the air reaches the lungs [15]. These different compartments allow for colonization of pathogens, as differences in temperature, mucus secretion, and relative oxygen concentration throughout the URT may regulate colonization, as it does in the gastrointestinal tract [16,17]. The vestibules contain stratified squamous epithelial cells that transition into pseudostratified epithelial cells, as well as hairs that are important for trapping large particulate matter [15]. Inspired air is heated and humidified within the turbinates of the nasal cavity, which also dehumidifies expired air. The nasal cavity has three turbinates that increase the surface area of the nose, and heat airflow entering the nasopharynx [18]. Glands and goblet cells are important for secreting the hydrated mucus layer, which contributes to humidifying incoming air and traps microparticles and microbes entering the URT from the environment. Finally, the humidified air enters the nasopharynx, which moves into the lungs for respiration within the alveolar space. Ciliated epithelial cells direct mucus flow toward the esophagus to remove trapped particles, which is known as mucociliary clearance [19]. Thus, the URT protects the lower airways from

temperature or particle-induced cellular damage. Air inspired into the URT impacts the inner ear since the Eustachian tubes contain air that is required for equalization of air pressure near the eardrum. Infections of the Eustachian tubes, known as acute or chronic otitis media, occur when pathogens evade physiological and immunological barriers of the URT [20,21]. Characterizing the microbiota of the deeper regions of the nasal cavity, including the sinuses, is challenging in routine clinical diagnostics; however, the more accessible regions of the nasal cavity have distinct microbiota profiles within an individual [22].

While the URT microbiota has long been recognized as a reservoir of pathogens, the lower respiratory tract has been thought to be sterile in healthy individuals. Studies from the 1960’s failed to recover viable bacteria in the lower airways of healthy individuals in bronchial swabs [23]. When bacteria or bacterial DNA were recovered from the airways it was believed to be due to sampling contamination by the upper airways [24]. Consequently, the lungs were one of the few body sites which were not sampled as part of the Human Microbiome Project [25]. Culture-independent approaches identified the presence of microbial DNA that originated primarily from the oral cavity [26,27]. The question of whether there was a unique microbial community in healthy adults was thoroughly investigated in studies that used extremely careful sampling techniques [27–30]. Neutral community modeling, which hypothesizes that the bacteria found in the lungs should match those found in the URT if it was contaminated, suggested that the microbial community of the lungs was distinct from that of the URT [30]. In chronic lower airway diseases such as cystic fibrosis, the lower airways are colonized by microbial communities clearly distinct from the URT [31].

An elegant study compared the regional colonization of bacteria in the oral cavity and established that there were three major niches in the mouth: the gingiva, buccal mucosa, and hard palate; saliva, tongue, tonsils, and throat; and the sub- and supragingival plaques [32]. The different locations had similar bacterial members but at different ratios: *Streptococcus* species were at the highest abundance in the gingiva, buccal mucosa, and hard palate, whereas the tonsils and throat contained the highest abundance of *Prevotella*. Dental plaques have low turnover rates compared to tissue surfaces with sloughing epithelial cells, promoting the growth of biofilms containing anaerobic bacteria including *Corynebacterium* species [32].

Bacterial spread from or within individuals is facilitated through air flow, nasal secretions, or saliva. Infections of the lower respiratory tract often originate

from bacteria in the oral cavity, in a process called 'microaspiration' [33,34]. Salivary excretions, which can be up to a liter a day, move bacteria from the tongue and throat/oropharynx toward the lower respiratory tract. Microaspiration is a frequent event, although contaminating commensal bacteria are cleared in healthy adults, and likely only cause disease in those with impaired lung or immune function [35].

The URT microbiota is also an important reservoir of potential lung pathogens, and determining if biogeographical niches exist for these pathogens may assist with disease prevention. Investigative studies that assess bacterial interactions between potential pathogens and other naturally colonizing members of the microbial community may lead to nonantibiotic-based methods of clearing pathogens. A recent study by Yan *et al.* elegantly assessed different regions of the nasal cavity that would be exposed to the external environment (anterior nares), as well as drainage from the sinuses (middle meatus and sphenoidal recess) in six *St. aureus* carriers and six noncarriers [22]. Comparing different regions along the nasal cavity, all sites contained similar relative abundance of Firmicutes, Bacteroidetes, and Actinobacteria, but the relative abundance of Proteobacteria varied throughout the URT. The anterior nares had the lower microbial diversity compared to the middle meatus and sphenoidal recess but the three sites selected had several unique operational taxonomic units (OTUs). When the *St. aureus* OTU was removed from the microbiota sequences, carriers could not be distinguished from noncarriers based on overall diversity. However, *St. aureus* carriers had high rates of *Corynebacterium accolens*, whereas noncarriers had high rates of *Corynebacterium pseudodiphtheriticum*, suggesting that some levels of niche competition could be important for colonization by *St. aureus* [22]. This was corroborated with *in vitro* data showing that *C. accolens* and *St. aureus* supported each other's growth, whereas *C. pseudodiphtheriticum* inhibited the growth of *St. aureus*.

Culture-based monitoring of URT microbiota

Nasopharyngeal carriage is a major contributor to infection, so the relationship between carriage rates, disease incidence, or antibiotic resistance is monitored by swabbing and culturing using conditions that enrich for specific pathogens. For example, *St. aureus* is often detected in greater than 30% of individuals, but rates of carriage of methicillin-resistant *St. aureus* (MRSA) vary widely from 3 to 30% [36–39]. The distinction between MRSA and methicillin-sensitive *St. aureus*

(MSSA) is important, as those colonized by MRSA are at an increased risk for invasive disease. A study by Davis *et al.* demonstrated that upon hospital admission, 21% of patients were MSSA carriers, and 3.4% of patients carried MRSA [40]; however, a much higher percentage of the MRSA carriers (19%) developed invasive staphylococcal disease than MSSA carriers (1.5%). Monitoring colonization may assist in the prevention of infection [40].

Nasal swabs have also been used to measure the efficacy of vaccination and to understand the interplay between age and environmental risk factors in carriage and disease risk [41,42]. For example, as a result of vaccination against *S. pneumoniae*, previously common serotypes which are included in the vaccine are being replaced with nonvaccine serotypes, and overall *S. pneumoniae* colonization has gone unchanged [43]. Surveillance studies have found that vaccination against *S. pneumoniae* also alters carriage of *St. aureus* and *H. influenzae* [43].

The limitation of these carriage studies is that they provide detailed data about specific pathogens but are less helpful in understanding the microbial community as a whole. Recently, a more in-depth culture-based study of the nasal microbiota assessed four locations along the length of the nose: the anterior and posterior vestibules, and the middle and inferior meatus [44]. In this study, 141 taxa, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species were found in four different sites in 34 surgical patients. The majority of species were found in all regions of the nose, except *Corynebacterium simulans*, which was not present in the posterior vestibule, and *Acinetobacter lwoffii*, which was not present in the anterior vestibule and inferior meatus. There were distinct pairs of species that were generally cultured together. For example, *Dolosigranulum pigrum* was cultured in samples that had *Corynebacterium propinquum* and *Staphylococcus epidermidis* was cultured in samples that had *Propionibacterium acnes*. This suggests that these pairs have a mutualistic relationship that may promote colonization or facilitate survival within the URT. Although this study provided the most extensive culture-based dataset on the composition of the URT microbiome, next-generation sequencing on a subset of samples, identified 113 bacterial phylotypes that were not recovered by culture. This highlighted that the extensive culturing methods used did not identify all the bacteria as they were able to detect more bacterial groups via next-generation sequencing [44]. However, culture-independent methods do not distinguish viable from non-viable bacteria, and some of the 'uncultured' organisms may not be viable in the samples.

Nonetheless, sequencing of the microbiota has become an important methodology for advanced bacterial composition assessment.

Culture-independent study of URT microbiota

The most common method for assessing the composition of the bacterial community is by sequencing the 16S rRNA gene. This gene has nine variable regions (V1–V9), differences in which allow for taxonomic identification of bacteria, which are classified as operational taxonomic units, or OTUs [45]. In general, targeting one or more of these variable regions is sufficient to identify members of the microbiota at the genus level, however, the specific 16S variable regions used may vary due to tissue type or particular bacterial families present [46]. While OTUs help quantitate the overall diversity of the microbiota, identification beyond the genera level can pose a significant challenge for determining exact community composition of the URT. As an example, the URT microbiota contains many members of the genus *Streptococcus* [47]. The high degree of 16S sequence similarity between this genus means that it is not possible to resolve the relative abundances of members of these genera [48,49]. Nonetheless, culture-independent techniques have been extremely useful in characterizing the composition and diversity of the URT microbiota. Sequencing of swabs of the middle turbinate, nasopharynx or anterior nares for nasal microbiota, and tongue or buccal mucosa for oral cavity sampling have identified that Corynebacteriaceae, Staphylococcaceae, and Propionibacteriaceae dominate in nasal cavity of healthy adults, although there is considerable compositional variability between the individuals [50]. The oral cavity in these healthy adults was dominated by Streptococcaceae and Veillonellaceae. The microbial communities from the three sampling sites were distinct from each other, signifying important local niches for colonization. Interestingly, a separate study determined that swabs are representative of invasive (surgical) sampling, suggesting that less invasive measures are adequate for nasal microbial study [51]. A summary of the most commonly found bacteria in healthy individuals are presented in Table 1.

Bacterial colonization, succession, and evolution in youth

Colonization of the URT begins at birth and, importantly, early colonization events impact respiratory health throughout life. Correlations between the method of delivery (i.e., vaginal or cesarean) and

breastfeeding with susceptibility to respiratory infections, asthma and allergy have been observed for decades [52–54]; it is now believed that this is due in part to the establishment of the URT microbiome. Recently, a study by Bosch *et al.* demonstrated that infants born via cesarean section were more likely to have reduced colonization levels of protective bacteria, including *Corynebacterium* and *Dolosigranulum* species [55]. Breastfeeding improves infant health in part because it facilitates the transfer of maternal antibodies which are then found in the infant's nasal secretions [56,57]. A recent study established the role of exclusive breastfeeding versus exclusive formula feeding from 6 weeks of age up to an age of 6 months of life on the URT microbiota [58]. Breastfed children had increased levels of *Corynebacterium* and *Dolosigranulum* species that may play an important role in protection against upper respiratory infections [14]. Furthermore, it was noted at 6 weeks, but not 6 months of age, that there was a negative correlation between wheezing and the relative abundance of *Dolosigranulum*. Infants colonized by *H. influenzae*, *Moraxella catarrhalis*, and *S. pneumoniae* within the first month of life were more likely to demonstrate wheezing, compared to those that were not colonized, but colonization status of these pathogens at 1 year of life did not correlate with wheezing [59]. A prospective cohort study involving 234 children demonstrated that nasopharyngeal colonization by *Streptococcus* at ~ 2 months of age was a strong predictor for asthma later in life [60]. This study also demonstrated that antibiotic use within 4 weeks of sampling increased the likelihood of colonization by *Streptococcus*, *Haemophilus*, and *Moraxella* [60]. The importance of the composition of colonizing bacteria in the first few months of life has been confirmed in mouse models of allergic asthma [61]. Mice treated with antibiotics prior to weaning had more severe allergic asthma, but antibiotic treatment after weaning had no effect [61].

Studies of microbial succession have been performed in order to understand how the development of the URT microbiota influences susceptibility to allergic airway disease. A recent landmark study by Biesbroek *et al.* investigated the bacterial succession patterns in infants [14]. The group followed a cohort of 60 infants over the first 2 years of life, sampling from 1.5 months of age to 24 months, and validated the findings with a cross-sectional study of 140 children per age group. The relative bacterial load and α -diversity did not change over time, however, the composition of the microbiota did change over time [14]. At 1.5 months of age, five clusters were observed that were dominated by either *Streptococcus*, *Moraxella*, *Staphylococcus*,

Table 1. Composition of the bacterial microbiota at different locations in the URT.

Population	Study	Sample site	Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria
Adult	[22]	Anterior nares	<i>Corynebacterium</i> , <i>Propionibacterium</i>	<i>Prevotella</i>	<i>Dolosigranulum</i> , <i>Staphylococcus</i> , <i>Streptococcus</i>	<i>Moraxella</i> , <i>Escherichia</i> – <i>Shigella</i>
		Middle Meatus	<i>Corynebacterium</i> , <i>Propionibacterium</i>	<i>Prevotella</i>	<i>Dolosigranulum</i> , <i>Staphylococcus</i> , <i>Streptococcus</i>	<i>Moraxella</i> , <i>Escherichia</i> – <i>Shigella</i>
Adult	[101]	Sinus	<i>Propionibacterium</i> , <i>Corynebacterium</i>	<i>Prevotella</i>	<i>Staphylococcus</i> , <i>Anaerococcus</i> , <i>Peptoniphilus</i>	<i>Ralstonia</i>
Children	[49]	Nasopharynx	<i>Corynebacterium</i> , <i>Propionibacterium</i> , <i>Bifidobacterium</i>	<i>Bacteroides</i>	<i>Staphylococcus</i> , <i>Faecalibacterium</i> , <i>Streptococcus</i>	<i>Moraxella</i>
		Oropharynx	<i>Rothia</i> , <i>Corynebacterium</i>	<i>Prevotella</i> , <i>Porphyromonas</i>	<i>Streptococcus</i> , <i>Veillonella</i>	<i>Haemophilus</i> , <i>Moraxella</i>
Adult		Nasopharynx	<i>Corynebacterium</i> , <i>Propionibacterium</i> , <i>Bifidobacterium</i>	<i>Prevotella</i> , <i>Sphingobacterium</i>	<i>Staphylococcus</i> , <i>Faecalibacterium</i> , <i>Streptococcus</i>	<i>Pseudomonas</i> , <i>Haemophilus</i>
		Oropharynx	<i>Rothia</i> , <i>Corynebacterium</i>	<i>Prevotella</i> , <i>Porphyromonas</i>	<i>Streptococcus</i> , <i>Veillonella</i>	<i>Haemophilus</i> , <i>Moraxella</i>
Elderly	[67]	Anterior nares	<i>Propionibacterium</i> , <i>Corynebacterium</i> , <i>Bifidobacterium</i>	<i>Prevotella</i> , <i>Bacteroides</i>	<i>Streptococcus</i> , <i>Staphylococcus</i> , <i>Veillonella</i>	<i>Moraxella</i> , <i>Pseudomonas</i>
		Oropharynx	<i>Propionibacterium</i> , <i>Corynebacterium</i> , <i>Bifidobacterium</i>	<i>Prevotella</i>	<i>Streptococcus</i> , <i>Staphylococcus</i> , <i>Veillonella</i>	<i>Moraxella</i> , <i>Pseudomonas</i>
Adult	[32]	Gingiva	<i>Actinomyces</i> , <i>Rothia</i>	<i>Porphyromonas</i> , <i>Prevotella</i>	<i>Streptococcus</i> , <i>Veillonella</i> , <i>Gemella</i>	<i>Haemophilus</i> , <i>Neisseria</i> , <i>Actinobacillus</i>
		Tongue	<i>Actinomyces</i> , <i>Rothia</i>	<i>Prevotella</i> , <i>Porphyromonas</i>	<i>Streptococcus</i> , <i>Veillonella</i> , <i>Granulicatella</i>	<i>Neisseria</i> , <i>Haemophilus</i>
		Supragingival plaque	<i>Actinomyces</i> , <i>Corynebacterium</i> , <i>Rothia</i>	<i>Porphyromonas</i> , <i>Prevotella</i> , <i>Capnocytophaga</i>	<i>Streptococcus</i> , <i>Gemella</i> , <i>Veillonella</i>	<i>Haemophilus</i> , <i>Neisseria</i> , <i>Actinobacillus</i>

Corynebacterium, or *Corynebacterium/Dolosigranulum*. In the *Streptococcus* and *Staphylococcus* clusters, *Corynebacterium* and *Dolosigranulum* were found at very low abundance. As the infants progressed from 1.5 months to 24 months of age, those whose microbiota were dominated by *Moraxella* were likely to remain dominated by *Moraxella*, and infants who were colonized with *Corynebacterium/Dolosigranulum* were likely to become dominated by *Moraxella*. In contrast, microbial communities dominated by *Streptococcus* and *Staphylococcus* were not stable and would often have a completely different profile in subsequent samples, with no defined pattern [14]. Interestingly, the *Corynebacterium/Dolosigranulum* cluster was highly correlated with breastfeeding. Furthermore, the children with a *Corynebacterium*- and/or *Dolosigranulum*-dominated URT microbiota were less likely to have a URT infection compared with all other microbial profiles. This study demonstrated a repeatable pattern of

ecological succession, and potentially identifies groups of infants that may be at risk for respiratory infections.

Another cross-sectional study assessed composition of the nasopharyngeal and oral microbiota in 51 young children and 19 parents by both culture and 16S rRNA sequencing [49]. Stearns *et al.* demonstrated that the oropharyngeal swabs had similar microbial communities regardless of age, and were dominated by *Streptococcus*, *Prevotella* and *Veillonella*. The adult nasopharyngeal samples were dominated by Firmicutes (including Lachnospiraceae, *Staphylococcus*, and *Streptococcus* species), Bacteroidetes (*Sphingobacterium* and *Prevotella*), and Actinobacteria (*Corynebacterium*, *Bifidobacterium*, *Rothia*, and *Propionibacterium*). In contrast, children were dominated by *Moraxella*, *Haemophilus*, Enterobacteriaceae, and *Enterococcus*. Targeted cultivation of these bacteria allowed for species identification, including *Staphylococcus*

(*St. epidermidis*), *Haemophilus* (*H. influenzae* and *Haemophilus parainfluenzae*), *D. pigrum*, and *Corynebacterium* (*C. durum* and *C. mucifaciens*) [49]. Furthermore, bacterial culture identified an increased bacterial density in the nasopharynx of children compared to healthy adults. This study demonstrated there is a dramatic difference between the infant and adult URT microbiota, suggesting progression to a more diverse, yet less dense, community.

Although the role of the URT microbiota in the immune system development is not as well described as the gut, there is some mechanistic evidence indicating that the microbiota in early life contributes to susceptibility to colonization by pathogens. A recent study demonstrated that macrophage recruitment to the URT during pneumococcal colonization was decreased in infant mice due to the inability to create a chemokine gradient within the nasal cavity [62]. In this model, the neonatal microbiota contributed to the high baseline chemokine expression, as antibiotic treatment decreased baseline chemokine expression, which allowed for a chemokine gradient to form upon pneumococcal exposure. Understanding the mechanisms by which the URT microbiota drives early innate immune development and susceptibility to colonization by pathogens will be an exciting avenue for future research.

Bacterial colonization and evolution in age

There are not many studies describing how the URT microbiota changes in older adults, but there is some evidence that age-related changes may contribute to the increased susceptibility to respiratory infections [63]. As an example, in children, colonization by *S. pneumoniae* occurs frequently and is generally asymptomatic; however, when colonization is not appropriately controlled, dissemination from the nasopharynx may result in pneumonia, meningitis, or septicemia [64]. In young adults, colonization is less frequent and of shorter duration due to adequate immune control, and consequently, disease is rare unless there are complicating comorbidities or influenza infection [65]. The dynamics of carriage in the elderly are not as well studied; however, as in adults, carriage rates are low [66,67]. The combination of low colonization rates and high incidence of pneumonia implies that colonization is brief and proceeds swiftly to infection [64]. In support of this, peaks of invasive pneumococcal disease in the elderly occur during Christmas holidays when contact with grandchildren, the major reservoir for *S. pneumoniae*, is presumed to occur [68]. Furthermore, mouse models indicate during

S. pneumoniae colonization the URT microbiota is profoundly distinct in aged mice, failing to return to a microbiota composition similar to prepneumococcal exposure, which younger mice were able to do [69,70]. Whether age-related changes in the microbiota contribute to permissiveness to infection is not clear, although preliminary studies support this hypothesis.

The nasopharyngeal microbiota of older adults appears to undergo profound changes. The anterior nares of adults (18–40 years old) is distinct from that of the oropharynx, but this distinction is lost in the elderly (> 65 years old) who become dominated by *Streptococcus*, *Prevotella*, and *Veillonella* [67]. This suggests the structure of the microbial community may degrade during aging, which facilitates an expansion of streptococcal species. As an example, Shannon diversity increases in elderly patients suffering from pneumonia compared to healthy elderly people [63]. The Shannon diversity Index is a metric which accounts for the abundance of different bacteria, with a low Shannon diversity suggesting the microbiota is dominated by a few bacterial species, whereas a high Shannon diversity suggests many different taxa of even relative abundance [71]. Pneumonia patients had a distinct decrease in anaerobic bacteria, including *Prevotella*, and decreased lactic acid bacteria *Leptotrichia*, and an increased viral load. A human experimental pneumococcal carriage model has identified that increased Shannon diversity in the URT microbiota correlates with a colonization permissive phenotype [72]. Mouse models of pneumonia have also implicated a role for dysbiosis, however, distinct microbial interactions that could be occurring have yet to be fully elucidated [69,70]. Whether specific bacterial species or overall microbial community dynamics are important for susceptibility to disease in the elderly is still unknown (Fig. 1).

Immunosenescence, which is defined as age-related changes in immune function, is a major contributing factor to the increased incidence of respiratory infections in people over 65 years old [73]. It is unclear how immunosenescence affects the composition and maintenance of the URT microbiota, although there are associations with the immune status and dysbiosis of the intestinal microbiota [74,75]. Impaired innate immunity in the URT has been described in elderly mouse models [76,77]. For example, TLR-1 expression, murine cathelicidin, and a decreased recruitment of macrophages in response to pneumococcal colonization contribute to decreased clearance of the bacteria in elderly mice [76]. More recently, tumor necrosis factor (TNF)- α was shown to be elevated in elderly mice, which promoted premature monocyte egress from the

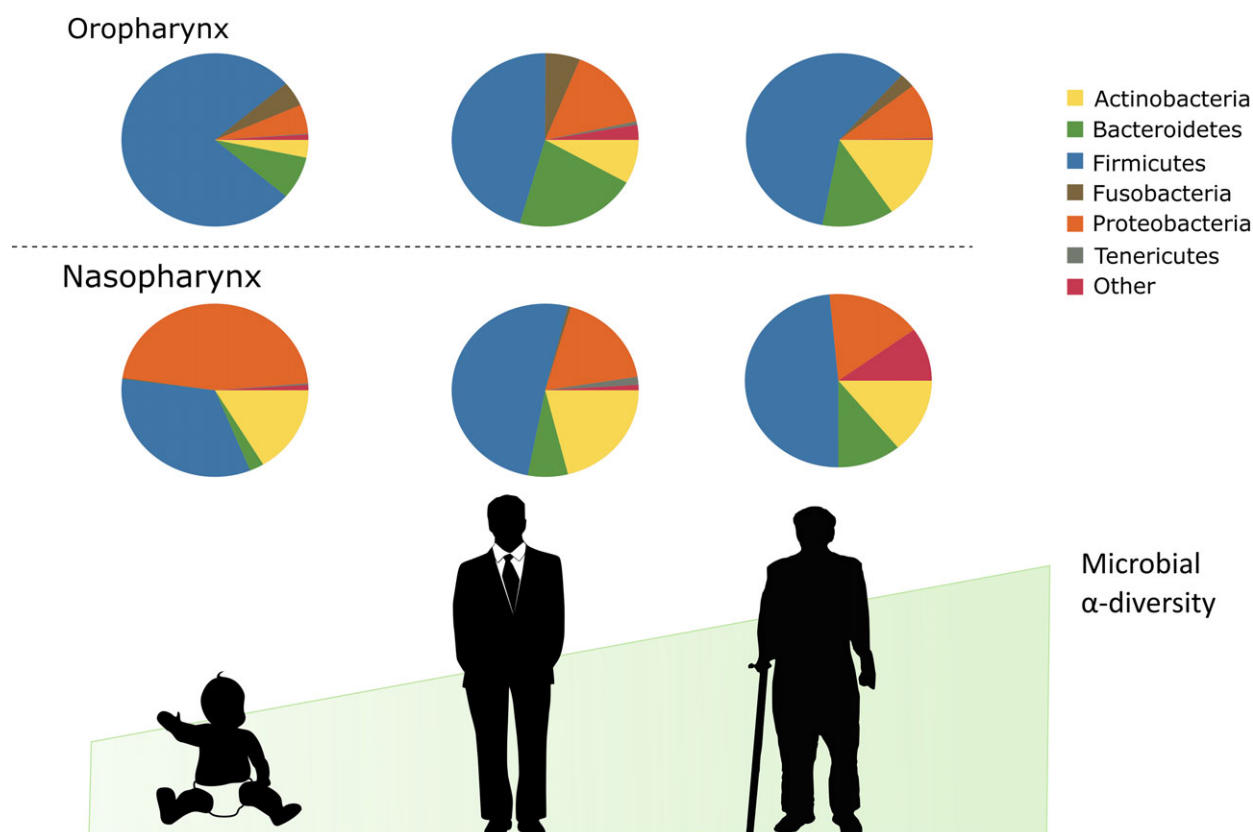


Fig. 1. The evolution of the upper respiratory tract microbiota during aging. The oral and nasal microbiome at birth and infancy is influenced by environmental exposures, including breastfeeding. The nasal and oral tissue sites provide unique niches for bacteria to grow and evolve, but by late age the nasal and oral microbiome become quite similar, revealing potential breakdown of important host mechanisms in microbiota community composition. This is highlighted by increased α -diversity (or microbial diversity within a host) during aging, indicating lack of community regulation.

bone marrow and impaired bacterial clearance upon reaching the nasopharynx [77]. These data correlate well with human data, demonstrating that elevated levels of circulating TNF- α and IL-6 are associated with increased risk for community-acquired pneumonia [78].

Factors affecting URT microbiota

Seasons

Seasonal patterns of respiratory infections are well documented. Community-acquired pneumonia is more prevalent during the winter and early spring months of the northern hemisphere [79]. Infants have the highest prevalence of *S. pneumoniae* nasopharyngeal colonization during cooler and drier months worldwide [80]. A number of factors have been proposed which may contribute to increased infection risk in the winter, including increased crowding or decreased immunity when exposed to colder air [81,82]. A study assessing

children < 7 years old found that seasonality affected both pneumococcal pneumonia and invasive pneumococcal disease [83]. Invasive pneumococcal disease increased in autumn, correlating with going back to school, whereas pneumonia cases increased in the winter [83]. Furthermore, these children carried a higher density of *S. pneumoniae* during the winter months. Health care professionals have asymptomatic carriage of various pathogens which differed between winter and summer, with high *M. catarrhalis* and Coronavirus in the winter and high *Klebsiella pneumoniae* in the summer [84].

Whether seasonal changes in the URT microbiota also contribute to infection risk is unclear. Bogaert *et al.* found an increased relative abundance of Proteobacteria during winter months and increased carriers of Fusobacteria and Cyanobacteria, although it is unknown whether this affected respiratory infections [85]. Infants with respiratory infections had increased carriage rates of *Haemophilus* in the spring–summer period and decreased *Moraxella* carriage during the

autumn–winter period [60]. Monitoring the changing dynamics within the URT microbiota may elucidate important interactions that occur in different humidity and temperature conditions.

Cigarette smoke

Exposure to cigarette smoke, whether by active or passive exposure, contributes to both chronic respiratory disease [e.g., asthma, chronic obstructive pulmonary disease (COPD)], and acute respiratory infections [86–88]. Cigarette smoke increases infiltration of inflammatory immune cells, and decreases mucociliary clearance in the upper and lower respiratory tracts, which ultimately leads to bacterial colonization and infection [86]. Smokers have increased microbial diversity compared to non-smokers [89]. Smokers have an increased likelihood of carrying pathogens, including *S. pneumoniae*, *Streptococcus pyogenes*, *H. influenzae*, and *M. catarrhalis* [90,91]. Consistent with this, smokers had less carriage of non-pathogenic *Streptococcus*, *Prevotella*, and *Peptostreptococcus* species, which have been shown to be inversely correlated with the presence of pathogens [90]. Smokers had a large increase in carriage of several gram-positive bacteria, including those associated with endocarditis and URT infections in the nasopharynx, which suggests that their increased risk of invasive disease is due to increased carriage of pathogenic bacteria [89]. Additionally, mice exposed to cigarette smoke concurrently with *S. pneumoniae* nasopharyngeal colonization had heightened invasive pneumococcal disease compared to room air-exposed mice, which was correlated with impaired innate immune responses [92]. This supports that the impairment of innate immunity and colonization resistance likely contribute to exacerbations of COPD. The oral microbiota has also been shown to be affected by cigarette smoke [29]. Smoking increases the relative abundance of *Megasphaera* species, associated with periodontitis, and decreases *Peptostreptococcus*, which has been shown to inhibit the growth of various URT pathogens *in vitro* [89,91]. Cigarette smoke has been demonstrated to affect bacterial adherence to oral tissue [93].

Cigarette smoke may also affect carriage and infection by altering bacteria directly. For example, biofilm formation and immune evasion increased in cigarette-exposed *St. aureus* [94,95]. Cationic antimicrobial peptides, including cathelicidin, rely on membrane charge to target and form pores through bacterial membranes [96]. Cigarette smoke extracts modulate the surface charge of *St. aureus*, thus reducing cathelicidin-induced lysis [95]. This effect is not limited to traditional cigarette smoke, as electronic cigarette smoke vapor increased virulence and biofilm formation,

decreased bacterial killing, and impaired epithelial and alveolar macrophage-induced killing of *St. aureus* [97]. Collectively, smoking has been demonstrated to affect multiple facets of the URT microbiota, including host defense, bacterial adherence and colonization, as well as pathogen virulence.

Chronic URT disease

Chronic rhinosinusitis is an inflammatory disorder of the sinuses which lasts for greater than 12 weeks. Microbial dysbiosis, defined as alterations of the microbial community believed to be associated with disease susceptibility or pathology, is a feature of chronic rhinosinusitis [98]. In contrast to other conditions (e.g., smoking, aging) in which there is an increase in microbial diversity, decreased microbial diversity has been reported in chronic rhinosinusitis patients [99–101]. This decreased diversity may be due to increased prevalence of anaerobic bacteria that are believed to thrive as a result of increased prevalence of anaerobic pockets which occur during biofilm formation [102–104]. The presence of *Corynebacterium* species correlates with optimal surgical outcomes, providing further evidence that *Corynebacterium* are beneficial members of the URT microbiota [101].

Genetic diseases affecting mucociliary clearance, such as cystic fibrosis and primary ciliary dyskinesia, affect the URT microbiota. Two recent studies have identified that infants with cystic fibrosis, which results in a thickened mucus layer, have an altered URT microbiota development compared to healthy infants including increased relative abundance of *Staphylococcus* species and decreased abundance of potential beneficial bacteria [105,106]. Many people with primary ciliary dyskinesia, a combination of diseases resulting in decreased ciliary action to clear mucus, also suffer from chronic rhinosinusitis, and there is a high reported rate of URT manifestations of disease, contributing to morbidity [107]. While no studies have been completed to assess the URT microbiota of these patients, the increased number of infections and antibiotic exposure are likely to alter the composition. Monitoring the URT microbiota of patients with impaired mucociliary clearance may result in decreased or prevention of lung infections in this susceptible population.

Intramicrobiota interactions

In addition to environmental factors and the immune status of the host, intramicrobiota interactions also influence the composition of the microbial community.

The majority of our understanding of competition comes from studies of pathogens, *St. aureus* and *S. pneumoniae*, which have a rich bactericidal arsenal which allows them to eliminate competitors in order to occupy specific niches. Although we have less information about how nonpathogenic members of the microbiota influence carriage, it is believed that they influence succession patterns, diversity, and infection risk [14]. Below we summarize some of the best characterized bacterial interactions which alter carriage dynamics in the URT, although we recognize that viruses and fungal species could also be playing a role in the URT microbiome.

Staphylococcus aureus* and *Streptococcus pneumoniae

The strongest evidence for interspecies competition between *St. aureus* and *S. pneumoniae* *in vivo* comes from studies of URT microbiota changes after pneumococcal vaccination. These studies demonstrate that as *S. pneumoniae* carriage in the nasopharynx decreases, carriage rates of *St. aureus* increase [43]. This is due in part to the bactericidal components produced by *S. pneumoniae*, such as hydrogen peroxide [65]. Hydrogen peroxide production induces DNA damage in *St. aureus* which triggers the release of resident prophages, resulting in bacteriophage-driven death of *St. aureus* [108]. Some strains of *St. aureus* produce catalase or carotenoids, such as staphyloxanthin, which reduce the damaging effects of peroxides and neutrophil killing [65,109]. These compensatory defense mechanisms and the fact that not all strains of *St. aureus* carry lysogenic phage, may explain why not all studies find an inverse relationship between *St. aureus* and *S. pneumoniae*. The immune status of the host also contributes to the apparent inverse relationship in carriage. Mouse models have shown that antibodies against *S. pneumoniae* dehydrogenase have cross-specificity for *St. aureus* which blocks subsequent *St. aureus* colonization [110]. Similarly, the reciprocal relationship between *S. pneumoniae* and *St. aureus* do not occur in HIV-positive children, which carry equal levels of both pathogens simultaneously, which implies that a fully competent immune system influences carriage [111].

***Staphylococcus aureus* and URT bacterial microbiota interactions**

Staphylococcus aureus and *H. influenzae* have a positive correlation with each other. Margolis *et al.* demonstrated that levels of *H. influenzae* were higher

when *St. aureus* was the initial colonizer of rat nasopharynx, possibly due to the liberation of nutrients by toxins released by *St. aureus* disrupting erythrocytes, including NAD and hemin [112–114]. However, *St. aureus* has an inverse relationship with several microbial community members [115]. After describing an inverse correlation between *Corynebacterium* species and *St. aureus*, Uehara *et al.* applied *Corynebacterium* to the nares of 17 *St. aureus* carriers. This resulted in the eradication of *St. aureus* in 71% of carriers, significantly more than those that received *St. epidermidis* or salt solutions, in a bacteriocin-independent mechanism [115].

Staphylococcus epidermidis, *Corynebacterium* species, and *St. aureus* occupy similar niches in the URT. Niche competition is controlled by the production of peptides, including bacteriocins, by different species which either impact viability or biofilm formation. Bacteriocins are normally pore-forming peptides produced by bacteria that target other, usually similar, bacteria. Some *St. epidermidis* strains produce an extracellular serine protease (Esp) that inhibits the biofilm formation and nasal colonization of *St. aureus* [116]. *St. epidermidis* Esp disrupts biofilm formation of several different strains of *St. aureus*, including those in coculture for over 1 year, suggesting that resistance does not develop. Esp-positive and -negative strains were placed in nasal cavities of *St. aureus* carriers, and only those colonized with Esp-positive strains saw reduction or eradication of *St. aureus*. Furthermore, Esp-induced disruption of *St. aureus* biofilms facilitated killing by human beta-defensin 2 in previously resistant strains [116]. The competition between *St. epidermidis* and *St. aureus* clearly involves both bacterial and host interactions.

***Streptococcus pneumoniae* and URT bacterial microbiota interactions**

Intense intraspecies competition is a feature of the *Streptococcus* genera, and is mediated in large part by bacteriocins. These peptides target similar bacteria, but usually are coexpressed with an immunity peptide to protect the producing bacteria [117]. A recent study assessed over 4000 *S. pneumoniae* genomes and found that there were over 250 unique combinations of bacteriocins across different strains, suggesting a very dynamic, complex interaction between strains [118]. Production of hydrogen peroxide by *S. pneumoniae* allows antagonism of many other bacteria, however, bacterial members of the URT possess other pathways to compete with *S. pneumoniae*. *H. influenzae* and *S. pneumoniae* cocolonize the nasopharynx in healthy

children, but competition is rampant between the two species. *H. influenzae* caused a rapid decrease in *S. pneumoniae* carriage within a day of cocolonization in immunocompromised mice [119]. Interestingly, recruited neutrophils were activated by peptidoglycan from *H. influenzae* to enhance killing of *S. pneumoniae* [119]. *S. pneumoniae* can impair fitness of *H. influenzae* and *Neisseria meningitidis* by producing neuraminidases that cleave sialic acids which these bacteria use to evade detection by the immune system [120].

In vitro studies have also suggested different bacteria can support or hinder *S. pneumoniae* growth. *S. pneumoniae* has the ability to respond to peptides from nasopharyngeal bacteria, including *Prevotella* species, which activate transcription of important colonization factors [121]. In contrast, *Corynebacterium* species seem to impair the persistence of *S. pneumoniae* [122]. Recently, it has been demonstrated that *C. accolens* have the ability to promote the killing of *S. pneumoniae* by metabolizing triacylglycerols, which are present on URT epithelium, to produce oleic and linoleic acids that are toxic to *S. pneumoniae* [123].

A human *S. pneumoniae* carriage model has been developed to assess relevant factors involved in colonization. Approximately 50% of those intranasally exposed to *S. pneumoniae* have successful colonization [124]. Establishment of pneumococcal carriage was not correlated with carriage of *St. aureus*, *M. catarrhalis*, or *H. influenzae*, although there were subtle decreases in *St. aureus* carriage over time [125]. Increased Shannon diversity increased likelihood of colonization, while *St. aureus*-dominated nasopharyngeal samples were less likely to be colonized [125]. Furthermore, successful colonization has been linked to increased viral carriage [126].

Conclusion

The URT is colonized by many different potential pathogens, and is constantly exposed to environmental bacteria, which compete for a niche for survival. Pathogens can survive and thrive in both the infant and elderly nasal and oral cavities, likely due to synergism between loss of colonization resistance and altered innate and adaptive immunity. Early colonization events, including birth route, breastfeeding, and exposure to other children influence the acquisition and succession of the URT microbiota, with *Corynebacterium* and *Dolosigranulum* species repeatedly demonstrated as protective profiles against disease [14]. Future work establishing early colonization events through culture-dependent and -independent methods, will help in the prevention of acute and chronic airway diseases

throughout the lifespan. Limited mechanistic studies have been completed to describe beneficial microbial colonization and interaction with the immune system in the URT. Recent work has identified that specific bacterial groups in the nasal cavity are associated with efficacy of vaccination against influenza, suggesting that nasal immunity may be regulated by the nasal microbiota [127]. Exploring host–bacterial interactions that have been uncovered in the gut, including adaptive immune development and pathogen clearance, could greatly decrease respiratory disease morbidity. Fecal transplants, which have been used to combat recurrent *Clostridium difficile* infections and diarrhea, have been identified to boost colonization resistance against the pathogen by altering the microbial community [128]. Colonization resistance can be caused by limiting nutrients essential for pathogen growth, disrupting the niche required by the pathogen, or enhancing the immune response by the body. Taking advantage of colonization resistance is only beginning to be explored in the URT. Bacterial and viral pathogens of the URT require unique niches, thus various microbial communities may be required to resist different pathogens. Development of human and animal models that assess the intramicrobiota competition and host–microbiota interactions are an exciting avenue in the development of targeted, URT-specific probiotics. The future of combating respiratory pathogens will require a thorough understanding of the dynamics of the URT microbiota and its' interaction with the host.

Acknowledgements

LPS is supported by a Canada Graduate Scholarship from the Canadian Institutes of Health Research (CIHR). MGS and DMEB are supported by the CIHR and hold Canada Research Chairs. Work in the Bowdish laboratory is supported by the McMaster Immunology Research Centre (MIRC) and the M.G. DeGrootte Institute for Infectious Disease Research (IIDR).

Author contributions

LPS, MGS, and DMEB all contributed to the writing and critical review of this manuscript.

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