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Integrating the microbiota of the respiratory tract with the unified airway model

Alissa S. Hanshew[a], **Marie E. Jetté**[b], **Sarah P. Rosen**[c], and **Susan L. Thibeault**[c,*]

[a]Environmental Health and Safety, 6 Eisenhower Parking Deck, The Pennsylvania State University, University Park, PA, USA

[b]Department of Otolaryngology, University of Colorado, 12631 E. 17th Avenue, Aurora, CO, USA

[c]Department of Surgery, Division of Otolaryngology-Head and Neck Surgery, University of Wisconsin-Madison, 600 Highland Avenue, Madison, WI, USA

Abstract

The unified airway model has developed from indications that the upper and lower respiratory tracts share key elements of pathogenesis. These shared traits likely extend to similar niche characteristics that support bacterial communities, and as such, we suspect that similar microbes exist on upper and lower respiratory tract epithelium. Over the past decade and a half there have been significant improvements in microbiological identification and analysis due to the development of new molecular technologies, including next-generation sequencing. In this review, we provide an overview of the modern collection and sequencing methods involved in respiratory microbiota research, and outline the specific microbial communities that have been found to be associated with the healthy and diseased human respiratory tract. Demonstration of a remarkable similarity between the upper and lower respiratory tract in terms of microbiological presence adds further corroboration to the existence of a unified airway.

Keywords

Respiratory; Bacteria; Microbiota

1. Introduction

Rhinitis and asthma share key elements of pathogenesis and have long been noted to co-occur, suggesting that the upper and lower respiratory tracts are more than just physically connected [1,2]. Indeed, the unified airway model suggests that immunological responses in one section of the respiratory tract can be linked to responses in other areas [3–5]. Data from the Human Microbiome Project (HMP) presents further supportive evidence of this model. Bacterial communities in healthy lungs have been shown to be highly similar to those in the upper respiratory tract, and shifts in one anatomical location may be associated with changes

*Corresponding author. 600 Highland Avenue, Madison, WI, 53792-7375, USA. thibeaul@surgery.wisc.edu (S.L. Thibeault).

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in others [6,7]. This is perhaps not surprising due to the ecologically similar niches found throughout the respiratory tract, many of which are covered in a continuous mucosal layer, and bathed in mucus and saliva.

Research in the past few decades has shown that many of the roughly 10^{14} microbial cells that live in and on our bodies are necessary for our wellbeing [8]. This consortium of microbes, termed the microbiota, serve a number of functions including priming the immune system, digesting food, providing nutrients and vitamins, and protecting us from potential pathogens [9]. Bacteria are estimated to outnumber human cells 10 to 1, and contain more collective genetic content than that found in human cells. Indeed, every surface of the human body in contact with the outside world is coated in microbes, including the gastrointestinal tract from the mouth to the anus, the respiratory tract from the mouth to the lungs, the entirety of our skin, even our eyes [6,7,10,11]. Interestingly, out of the about 50 known bacterial phyla, humans generally only associate with members from ten of these phyla, including Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes, TM7, and Verrucomicrobia [12].

The methods used to study these bacterial communities has changed drastically in the past decade, due in large part to a vast increase in computing power coupled with the advent of new molecular technologies, often called “next-generation sequencing” (NGS), that generate thousands to millions of sequences per sample [13]. While standard culture-dependent work certainly laid the groundwork for this field of research, NGS has opened the door to a better understanding of the breadth and depth of the human microbiota. Understanding what grows attached to surfaces of the human body may give us not only a better view of what it means to be healthy, but also open avenues to a better understanding of chronic diseases that may be associated with an altered microbiota, and may aid in development of better treatments and perhaps even preventative measures.

The goals of this review are three-fold: 1) give a brief overview of how human microbiota research is conducted in general and specifically in the respiratory system; 2) review the microbial community found associated with the healthy and diseased human respiratory tract; and 3) discuss how this corroborates the unified airway model.

2. Human microbiota – modern sequencing methods

Study of the human microbiota starts with sample collection. Samples may be collected in many different ways, depending on the site being studied and the questions researchers are trying to answer. Swabs and brushes have both been used to physically remove bacteria from any surface the implement can reach, along with instruments that more literally scrap the epithelia. Likewise, liquids (saliva, sputum, vaginal secretions, and gastric juices) and solids (feces) can be used. Historically, and still today in many labs, these samples were used in culture based assays, where an attempt was made to grow bacteria on agar plates or in liquid media, and then identify the bacteria present in the original sample. However, today we know that many bacteria associated with humans are not easily cultured, and this older method of sampling missed the vast majority of bacteria associated with humans [14]. The inability to culture bacteria from some areas of the body, such as the stomach and lungs, led

to the belief that these sites were sterile. This idea has been shown to be incorrect, though many of these bacteria remain elusive in terms of required culturing conditions [14].

The discovery of the 16S rRNA (16S) gene and the advent of polymerase chain reaction (PCR) birthed a new era in microbial ecology research. Pioneering work by Carl Woese demonstrated that distinct groups of bacteria could be identified based on the 16S gene [15]. The 16S gene, universally present in all bacteria, is about 1500 base pairs long and contains nine hypervariable regions flanked by highly conserved regions (Fig. 1). This gene structure is highly amenable for identifying bacteria; universal primers can be designed for the conserved regions, while the intervening regions can be used for sequencing and identification of bacteria. Out of this came a number of techniques to explore microbial communities using molecular techniques, such as clone libraries, denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (tRFLP), amongst others [16,17]. The drawbacks of these techniques have made them less popular, and they have mostly been replaced by newer sequencing technologies.

The advent of NGS techniques, so named because they further the ideas of Sanger sequencing, made possible the description and exploration of microbial communities like never before [18,19]. Where before a successful clone library may have yielded a few hundred clones from a handful of samples, we can now recover millions of sequences, easily multiplexed across numerous samples. Sequencing platforms such as the MiSeq and HiSeq from Illumina, and SMRT sequencing from Pacific Biosciences, in addition to others, make this possible. In short, for bacterial community analysis using the 16S gene, PCR products are produced targeting relatively short regions of the 16S gene using universal bacterial primers that also contain sequences specific for the sequencing platform being used. Primers are usually also designed with barcodes for each sample allowing each sample to be labeled with a unique 4-12 bp DNA fragment that, after sequencing, can be tied back to the original sample [20]. This also allows PCR products from numerous samples to be pooled, sequenced en masse, and the sequences for each sample to be separated later based on the assigned barcode using bioinformatics programs such as mothur or QIIME [21,22].

These current sequencing techniques are not without bias. Results from experiments may vary based on the sampling technique, DNA extraction protocol, polymerase used for PCR along with the primer choice and region of the 16S gene sequenced [23–25]. Likewise, multiple protocols and programs exist for processing sequences [21,22], checking sequence quality and removing noise [26,27], and detecting chimeras [28,29]. Sequence identification can also vary, as the array of databases for identification purposes each have their own strengths and weaknesses [30–32]. Even how sequences are aligned to these databases is important [33]. Each step in the overall protocol for how samples are handled introduces bias and studies done using different protocols are not always easily comparable to each other.

3. Sampling the respiratory system as a niche for bacteria

For the purposes of this review, we use the term ‘respiratory tract’ loosely to include all epithelial surfaces associated with respiration and the path air takes to reach the lungs,

including surfaces from the anterior nares into the nasal cavity and sinuses, back to the nasopharynx, the soft tissue of the oral cavity, back to the oropharynx, down through the larynx, trachea, and finally, to the lungs (Fig. 2). Two types of epithelia dominate these surfaces. Ciliated pseudostratified columnar epithelium lines the sinuses, nasal cavity, nasopharynx, larynx, and trachea, while the oral cavity, oropharynx, and vocal folds are lined with stratified squamous epithelium. Mucin producing goblet cells are found only in ciliated pseudostratified columnar epithelium, while submucosal glands found throughout the respiratory tract are also known to produce mucus.

Mucin, a heavily O-glycosylated protein, is found in both saliva and the continuous mucus layer that covers all epithelial surfaces of the respiratory tract. It likely serves as the primary source of nutrients for bacteria in the respiratory tract [34]. Multispecies bacterial communities are known to digest mucin, often in cooperation using overlapping patterns of enzyme activity [35]. Partial consumption by bacteria of mucins, which have no known antibacterial activity, suggests that part of their physiological function is to serve as nutrients for bacteria [34]. Saliva, despite containing antimicrobial compounds such as lysozyme, lactoferrin, lactoperoxidase, and IgA, also supports bacterial growth [36]. In addition to nutrients, mucus attachment to the epithelial surface provides an adhesion site for bacteria, decreasing the rate at which they are eliminated from the respiratory tract despite mucociliary beating that is generally accepted to move mucus out of the respiratory system.

Many surfaces in the respiratory tract are highly amenable to sampling for bacterial community analyses, including those in the oral cavity, nasopharynx, oropharynx, anterior nares, and nasal vestibule. Design of the Human Microbiome Project (HMP) was particularly cognizant of this, and chose to focus on seven easy to sample sites to describe the bacterial community of the oral cavity and respiratory system including the tongue dorsum, hard palate, buccal mucosa, keratinized gingiva, palatine tonsils, oropharynx, and saliva [37]. Conversely, sampling from other sites of the respiratory tract, such as the lungs, sinuses, and vocal folds are considered invasive. Large-scale studies, like the HMP, for these sites in the respiratory system remain rare due to these limitations.

Collection of samples in the respiratory tract can be taken in a number of ways. Swab and brush sampling can be done on any location that can be reached while minimizing damage to the epithelial surface. Additionally, fluids such as saliva and induced sputum have been used for many studies, as have washes from locations such as the oral cavity, sinus cavities, and lungs. However, cross contamination in sites such as the lungs can be problematic, where the bronchoscope required for sampling may contaminate the lower respiratory system with bacteria from the upper respiratory system. Charlson *et al* developed a two bronchoscope approach to address this concern, where samples taken from the glottis with the first bronchoscope were used to determine what, if any, contamination a bronchoscope might transport to the lungs, and then a second bronchoscope was used to take samples from the lungs [7]. While bacterial numbers were lower in the lungs, cross contamination did not appear to be a problem. Likewise, those sampling in the sinuses have noted awareness of preventing nasal community contamination when withdrawing the sampling device [38,39].

Additional questions remain about how well swabs and brushes remove bacteria that are well attached to epithelial surfaces. While swabbing to collect microbes is highly convenient, few studies have compared the resulting community from easily removed bacteria versus those more firmly attached. Research in the ileal pouch suggests that brush sampling is sufficiently similar to samples taken via biopsy [40].

4. Healthy human respiratory microbiota and the unified airway model

The unified airway model suggests that different areas of the respiratory tract share many similar characteristics [3]. These shared traits likely extend to similar niche characteristics that support bacterial communities in similar mucosal surfaces, and some have even suggested that the respiratory tract should be viewed as a single ecosystem [41]. Shared traits such as a continuous mucosal layer, exposure to inhaled air, and nutrient availability in the form of mucin, may support the growth of similar microbial communities, while the differences seen may be due to local characteristics such as increased presence of saliva, exposure to ingested substances, proximity to skin surfaces, or increased oxygen concentration. Despite the methodological problems of comparing studies done using different protocols, and ethical issues of invasively sampling healthy subjects, both discussed above, a broad view of what constitutes the healthy microbial community in the respiratory tract is emerging (Table 1).

In the largest study to date, roughly 300 healthy individuals were recruited for an in depth study by the Human Microbiome Project (HMP), a large multicenter study funded by the National Institute of Health [42]. Due to the stringent exclusion criteria [37], data from the HMP provides a large framework for what the healthy human microbiota constitutes at seven sites associated with the respiratory tract (described above). These sites could be characterized by two distinct, but similar, bacterial communities that differed more in the abundance of community members rather than what taxa were present in the community [6,43]. While there is little agreement on whether a “core” microbiota exists in any body site, *Prevotella*, *Streptococcus*, and *Veillonella* were consistently present in the HMP subjects across all sites tested in the oral cavity and oropharynx [6]. Segata *et al* surmised that the similarities in the bacterial communities from these seven body sites may be due to the buffering nature of saliva and high nutrient availability in the form of mucin [6]. Recent reanalysis of this data using oligotyping, a high resolution method that looks at very fine sequence differences, suggests that a core oral community may indeed exist [44,45]. Eren *et al* found 58 oligotypes in 95% of HMP oral samples sequenced using V3-V5, suggesting a highly similar community across these subjects [45]. Many of these oligotypes were identified as taxa found in the original data set including *Neisseria, Streptococcus*, and *Veillonella*.

Smaller studies corroborate both the original data from the HMP and the oligotype reanalysis. Charlson *et al* described highly similarly communities in the nasopharynx and oropharynx of 33 healthy nonsmokers [46]. Communities in the nasopharynx were distinguished by *Corynebacterium, Propionibacterium*, and *Staphylococcus*, taxa also associated with the skin [47], while the oropharynx was distinguished by *Fusobacterium, Haemophilus, Neisseria,, Prevotella*, and *Veillonella* [46]. Despite these differences, many

shared taxa were also present including *Lactococcus, Leuconostoc, Streptococcus*, and *Veillonella*. A study by Jette *et al*, which analyzed false vocal fold tissue biopsies from 97 healthy individuals, identified that the most common bacterial communities in the larynx are unclassified genus of *Comamonadaceae, Streptococcus, Cloacibacterium, Prevotella, Propionibacterium, Helicobater*, and *Veillonella* [48]. Follow up work by Charlson *et al* expanded their sampling of the naso and oropharynx to the lungs, a notoriously difficult location to sample without cross contamination with bacteria from the upper respiratory tract as discussed above. Lung samples from six healthy patients were indistinguishable from upper respiratory sites such as the oropharynx, nasopharynx, and oral washes [7]. Most samples, regardless of sample location (oral versus lung), or technique (swab versus wash) were dominated by community members in the families Prevotellaceae, Streptococcaceae, and Veillonellaceae. Like their previous work, nasopharyngeal samples also had higher abundances of bacteria often associated with the skin, including members of the families Corynebacteriaceae, Propionobacteriacae, and Staphylococcaceae. Morris *et al* further confirmed the ubiquity of *Streptococcus, Veillonella*, and *Prevotella* in oral washes and bronchoalveolar lavage (BAL) in a larger cohort (n = 45) of healthy nonsmokers [49].

However, study of healthy patients remains problematic and limited. Parameters used by the HMP would exclude many patients used as healthy controls in other studies. While Jensen *et al* found the same genera as mentioned above present in their healthy subjects in a tonsillar crypt study, these patients were also undergoing surgery for removal of vocal fold polyps and a benign tumor in the throat, conditions that would exclude these patients as healthy in the HMP [37,50]. Likewise, similar community structures have been found in the middle meatus and maxillary sinus, but control populations in these studies would also not meet the criteria for healthy in the HMP due to pituitary tumor [51], obstructive sleep apnea [52], and fungal ball [53].

Despite these challenges, and the question of who is healthy enough to include as healthy subjects in these types of studies, *Prevotella, Streptococcus, and Veillonella* have been found as prominent community members in all respiratory sites except the anterior nares (Table 1). This is perhaps not surprising since the nares have different niche characteristics which is likely reflected by the similarity of their microbiota to skin [12]. It is likely that characteristics of these genera allows them to flourish in the respiratory tract while being rare in other locations such as the gastrointestinal tract [6].

5. Microbiota of the diseased respiratory system

While description of the microbial community of the healthy respiratory tract is indeed interesting, the comparison to communities found in diseased patients has begun to offer insights into factors affecting disease that were not even considered until about a decade ago. The first glimpses that microbial communities might affect human health came from studies comparing obese and lean twins, which showed that despite identical genetics, obesity could be correlated to changes in the microbial community of the gastrointestinal tract [61]. Likewise, alterations in the diversity of microbial communities have been identified within the respiratory tract of smokers compared to non-smokers [46,48], and disease processes such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and asthma.

Cystic fibrosis is perhaps one of the best studied diseases affecting the respiratory tract in terms of bacterial community composition and shifts that may be associated with health and disease. Traditionally, deaths from infection in this patient group have been associated with a handful of opportunistic pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, numerous groups over the past few years have clearly demonstrated that cystic fibrosis lungs are microbially more complex than previously thought, much like the diverse array of bacteria that can be found in healthy lungs. Taxa well known to inhabit the healthy respiratory system also inhabit patients with CF, such as *Gemella, Prevotella*, *Porphyromonas*, *Rothia*, *Stenotrophomonas*, and *Streptococcus* [54]. Likewise, the diversity of bacteria found associated with the lungs of patients with CF is higher than previously suggested by cultureand cloning-based studies [54–56]. Additionally, recent work has shown that decreases in bacterial diversity in the lungs, specifically reductions in *Gemella, Granulicatella, Prevotella, Streptococcus*, and *Veillonella*, were highly correlated with poor lung function [57].

Nasal communities were also highly similar in healthy study participants and patients with COPD and asthma, regardless of health status [58]. Though this study used clone libraries with the acknowledgement of likely undersampling the community, asthmatic lungs were highly different from healthy lungs, though their oropharynx communities were not, while both the lung and oropharynx samples from COPD patients were markedly different from healthy. Increases in *Haemophilus* in both COPD and asthmatics coupled with a decrease in *Prevotella* seem to explain most of these differences. Further work in patients with moderate and severe COPD showed decreased community diversity compared to smokers, healthy never smokers, and two patients with mild COPD [59]. Despite this, *Fusobacterium, Haemophilus, Porphyromonas, Prevotella, Pseudomonas, Streptococcus*, and *Veillonella* were still common community members. Explanted lungs from COPD patients, while dominated by *Pseudomonas*, also showed microanatomic heterogeneity, much like every other body surface that has been sampled [59–61].

Similar shifts in community membership have been found in other respiratory maladies. Recurrent tonsillitis in adults was associated with increases in *Fusobacterium necrophorum, Streptococcus intermedius*, and *Prevotella melaninogenica/histicola*, while pathogens traditionally thought to be the etiological agent were not found, such as *Staphylococcus aureus* and *Streptococcus pyogenes* [50]. Likewise, rhinosinusitis was shown to be associated with a decrease in lactic acid bacteria coupled with an increase in *Corynebacterium tuberculostearicum*, a bacterium generally considered to be a member of the skin microbiota [52]. While sampling healthy vocal folds remains problematic, benign vocal fold lesions were found to have a significantly higher abundance of *Streptococcus pseudopneumoniae* compared to false vocal fold biopsies [48,62].

6. The unified airway and implications of the microbiota in respiratory diseases

The unified airway model suggests that disease in one part of the respiratory tract may be associated with disease in distal locations. One might hypothesize that taking, perhaps, an

oropharynx swab could indicate shifts in the microbial communities in other regions of the respiratory tract and serve as a marker for disease. While healthy communities in the lungs are highly concordant with samples from the upper respiratory tract, studies matching disease in one location to samples in others remains scant.

Goddard *et al* found that while sputum samples matched lung samples in cystic fibrosis patients, throat swabs were discordant [56]. However, this study was only based on three patients, and as the HMP has demonstrated, larger numbers are needed to see the overall patterns of microbial communities. In a larger study, the exact opposite conclusion was reached, where the lungs of healthy smokers (n = 19) had highly similar communities compared to oral washes [49]. Large scale studies are needed to determine if this type of noninvasive sampling is indeed a sufficient representative sample for other locations in the respiratory tract. To date, most studies in the respiratory tract of diseased individuals rely on a single sample type or do not include a large enough patient population to determine this.

7. Future directions in respiratory microbial research

Large knowledge gaps remain in our understanding of the composition and function of the microbial community associated with the respiratory tract despite these large leaps in research in the last decade and a half. While the respiratory tract is composed of many smaller niches characterized by different cell types, nutrient and oxygen availability, and the presence of saliva and mucous, the similarity between communities in these locations is remarkable. The ubiquity of species in the genera *Prevotella, Streptococcus*, and *Veillonella* speaks to their ability to survive in respiratory niches despite variable conditions.

Research is still needed to tie together the complete picture of what a healthy microbial community can look like in the respiratory system. Large scale studies like the HMP are required for the respiratory tract before we can have a full grasp of the range of community composition that may constitute healthy. Work in the gastrointestinal tract has shown that no single community can be described as healthy, rather these healthy communities may have completely different composition while still serving the same function [12]. Likewise, additional research is needed to tease out whether perturbations in one respiratory niche are related to responses and changes in other areas as noted in the unified airway theory, and particularly if they are associated with disease. Current research has largely focused on localized responses to changes in community membership and structure. These changes could ripple out in systemic responses and changes in even further distal locations of the body, such as the gastrointestinal tract and skin, may be more interrelated than currently thought.

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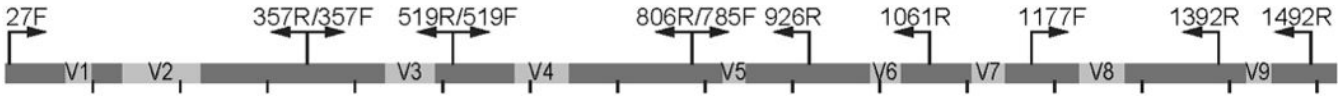

Fig. 1.

Basic structure of the 16S rRNA gene found in all bacteria. The gene product of the 16S rRNA gene serves a structural role in the 30S small subunit of all bacterial ribosomes. The 16S gene is roughly 1500 base pairs long, and characterized by nine hypervariable regions, marked as V1-V9 in light gray, dispersed between highly conserved regions, denoted in dark gray. Hash marks are placed every 100 base pairs, and the diagram has been drawn to scale. Useful in identifying bacteria, primers can be designed for these highly conserved regions and the intervening sequences used for identification of bacteria. Commonly used primers are noted in the figure including arrows noting the direction they are used for amplification in PCR. Choice of primer pair, one forward and one reverse, is determined by a number of different options. Particularly important is production of an appropriate length amplicon for the chosen sequencing platform. Illumina platforms such as the HiSeq and MiSeq require shorter amplicons, such as those produced from using 519F and 806R. Primers are named based on their base pair position in the *Escherichia coli* 16S gene, with F for forward primers and R for reverse primers.

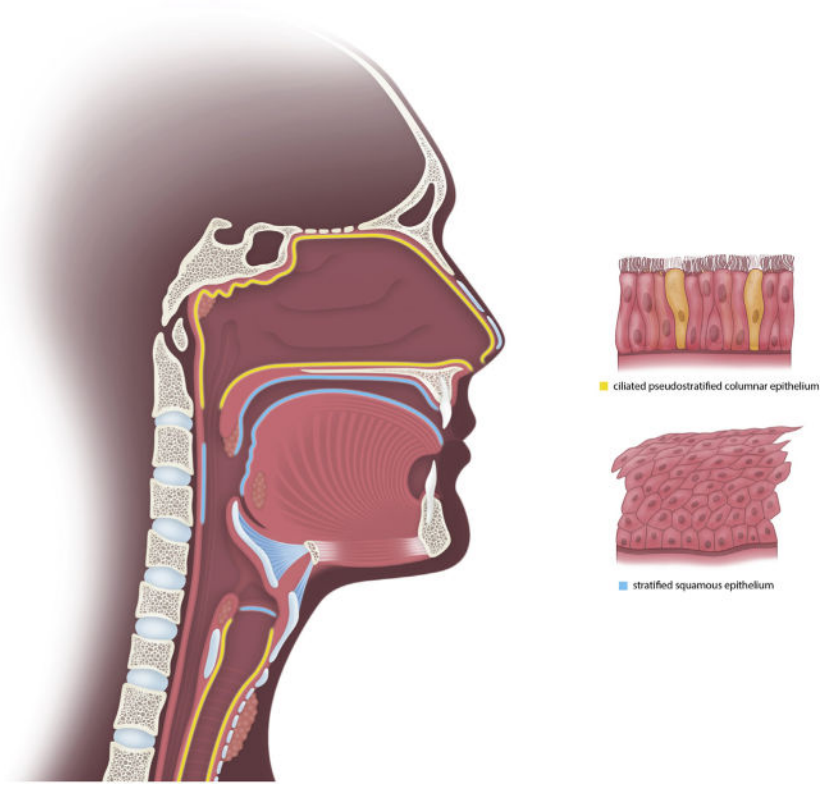

Fig. 2.

Illustration of the basic anatomy of the respiratory system with the corresponding epithelial types for each anatomical site. Ciliated pseudostratified columnar epithelium (yellow) is found in the sinuses, nasal cavity, nasopharynx, larynx, and trachea. Stratified squamous epithelium (blue) lines the oral cavity, oropharynx, and vocal cords.

Table 1

Table of common microbiota found within the regions of the respiratory tract.

Oral cavity	Nasopharynx	Oropharynx	Larynx	Lung
Prevotella	Corynebacterium	Prevotella	Prevotella	Prevotella
Streptococcus	Streptococcus	Streptococcus	Streptococcus	Streptococcus
Veillonella	Veillonella	Veillonella	Veillonella	Veillonella
Haemophilus	Propionibacterium	Haemophilus	Unclassified-Comamonoadaceae	Pseudomonas
	Staphylococcus	Fusobacterium	Cloacibacterium	Fusobacterium
	Moraxella		Helicobacter	