Respiratory Microbiota and Lower Respiratory Tract Disease

Introduction: The respiratory airways harbor a complex succession of ecological niches with distinct but related bacterial communities. Particular challenges of respiratory microbiome research have led to limited scientific output compared to other human microbiomes.

Areas covered: In this review, we summarize the current state of knowledge of the bacterial respiratory microbiome, with a particular focus on associations between the respiratory microbiome and lower respiratory tract conditions.

Expert commentary: There is growing evidence that the respiratory microbiome is associated with lower respiratory infectious diseases and related conditions. Most respiratory microbiome reports are metataxonomic cross-sectional or case-control studies with relatively small sample sizes. Large, prospective projects with metatranscriptomics or metabolomics approach are needed to unravel the effect of the respiratory microbiome on health-related conditions. Moreover, standardization in sampling, library preparation, sequencing techniques and data analysis should be encouraged.

1. Introduction

In the past decade, we have witnessed an exponential increase in human microbiome related research, from around 300 publications in 2006 to almost 8000 in 2016. The development of novel culture-independent techniques of microbial identification, and decreasing costs of sequencing platforms have enabled research in this exciting field, producing relevant findings that have changed and broaden our perspective on host-microbe and microbe-microbe interactions. Important paradigm shifts have occurred during this process, including the recognition of the diversity and abundance of the microbial community in healthy individuals, the discovery of viable communities in body sites previously considered sterile, and more importantly, the establishment of associations between human microbiome and health.

Most of the research conducted so far has focused on the gut microbiota, the body site containing the richest and most complex bacterial communities. In 2008, the National Institutes of Health and the European Commission launched two multicentric mega projects: the Human Microbiome Project, where samples from up to 18 body sites of healthy individuals were collected, and MetaHIT, focused on the metagenomics of the intestinal tract.[1,2] The respiratory tract was left apart, partly because of problems in collecting lower respiratory tract samples, but also because lungs have traditionally been considered sterile. Recent studies have shown that the lower respiratory tract harbours a diverse bacterial community both in healthy and diseased individuals, which has boosted respiratory microbiota research that is currently growing rapidly.[3]

Acute lower respiratory infections, such as pneumonia and bronchiolitis are a major public health concern globally, accounting for a large burden of mortality and hospitalization in children.[4-6] Chronic conditions, such as asthma and chronic obstructive pulmonary disease (COPD), show a high and growing prevalence globally, with respiratory infections being involved in pathogenesis and course of the disease.[7-10] Respiratory microbiota research may reveal new perspectives regarding host-microbe interactions leading to a particular local environment contributing to the pathogenesis of lung and airways conditions. These deeper insights could later be the basis for novel preventive and therapeutic measures to complement or adapt the currently available arsenal against respiratory infectious diseases.

Besides an initial overview of respiratory microbiota composition and dynamics, this review aims to summarize published evidence of associations between respiratory microbiota and different respiratory conditions.

2. Terminology and methods

Most microbiota studies rely on sequencing variable regions of the highly conserved gene encoding the 16 small subunit of ribosomal RNA (16SrRNA). This gene is universally present in bacteria and absent in mammals, and contains 9 hypervariable regions (V1 to V9) allowing the identification of different bacteria through taxonomic assignment of the obtained sequences using reference genomes.[11] Depending on the sequences and the available databases, identification can be achieved to the species level but usually results in a mix of species, genera, phylum, and taxa levels. To

describe the output of microbial identification, the generic term organizational taxonomic unit (OTU) is usually adopted. This process is defined as metataxonomics as it describes the taxonomic composition of the entire bacterial community. This has two important implications: first, viruses and fungus (which lack the 16S rRNA gene although similar profiling methods based on the 18S rRNA gene are available for fungus) are not identified albeit being an integral part of the microbiota, and second, this process does not provide information about the function of the microbiota, which can only be gained through metagenomics or other omics platforms, such as metatranscriptomics and metabolomics. Key terms used in microbiota research are defined in Table 1.

3. Ecological niches and dynamics of the respiratory microbiota

3.1. Microbiota acquisition of the upper respiratory tract

From a bacterial ecological perspective, the human respiratory tract is a sequence of niches from the anterior nares and mouth to the lungs harbouring distinct but related bacterial communities. There is recent evidence of foetal microbial colonization starting in utero,[12,13] with intensification of the process immediately after birth. Maternal bacteria rapidly colonize cutaneous and mucosal surfaces of the newborn, with predominance of vaginal and intestinal maternal microbiota in case of vaginal delivery, and of skin microbiota in births resulting from caesarean sections.[14] The anterior nares display a relatively simple microbiota with few differences between children and adults. *Corynebacterium*, *Propionibacterium* and *Staphylococcus* are the initial colonizers of the anterior nares and maintain their predominance until adult life where *Moraxella* has also been described together with the three genera above.[15] In contrast, nasopharyngeal microbiota is a more complex and dynamic ecosystem with important changes over time. Differentiation into site-specific microbiota is a multifactorial process that starts after initial colonization and can last to up to two years of age depending on body sites, with the upper respiratory tract microbiota of children 18 months of age still showing distinct characteristics compared to that of adults.[16,17] Initially, *Corynebacterium* and *Staphylococcus* dispersing from the anterior nares colonize the nasopharynx that subsequently gains *Dolosigranulum* (particularly in breastfed infants), *Alloiococcus* and *Moraxella*.[18-20] In adults, the inter-individual diversity in nasopharyngeal microbiota is reduced, *Corynebacterium* is usually the dominant genus, and *Moraxella* and *Dolosigranulum* are much less frequently represented.[21-23] The oral cavity shows the same initial colonization strongly influenced by delivery mode as the other body sites, but rapidly gains a higher richness due to a high exposure to the external environment, the presence of saliva characterized by abundance of *Prevotella*, *Neisseria*, and *Haemophilus*, and later on, by the eruption of teeth entailing an increase in *Bacteroidetes*, particularly *Veillonella* and *Prevotella*.[24] Despite being highly exposed to foodborne and environmental bacteria, the oral microbiota is remarkably stable over time.[25] The oropharynx is an anatomical crossroad between the upper respiratory structures, and the lower airways and gastrointestinal tract resulting in a rich and highly variable bacterial ecosystem. Common genera described in healthy adults include *Streptococcus* (both commensal and potentially pathogenic such as *S. pneumoniae* and *S. pyogenes*), *Haemophilus*, *Neisseria*, *Prevotella*, *Veillonella* and *Leptotrichia*.[26,27] Similar to what happens in the

gut mucosa and skin surface, the early colonization of upper airways plays an important role in immune maturation and immune response modulation of the host, as suggested in studies where germ-free mice showed exaggerated airway eosinophilia and accumulation of invariant natural killer T-cells that did not occur in colonized mice.[28-31] Initially, the neonate immune system is skewed towards a regulatory response allowing microbiota colonization without inflammatory response. The risk of having an immature immune system at birth is rapidly compensated by the beneficial effect of having a stable commensal microbiota conferring resistance to colonization by pathogenic bacteria. The contact between resident microbiota and the infant immune system is limited by physical barriers such as mucus layers, and modulated by metabolites, microbes, and IgA contained in maternal milk, leading to immunogenic tolerance and the establishment of a stable host-microbiota relationship.[32] Despite the virtually absent inflammatory response against stable microbiota, the constant exposure to limited amounts of bacterial antigens contributes to the development of the immune system.

3.2. The lower respiratory tract microbiota

The healthy lung sterility paradigm has repeatedly been challenged since the introduction of culture-independent techniques. However, this does not imply the existence of stable colonization of the lungs and it is commonly accepted that bacterial detection in lower airways samples is likely due to equilibrium between constant inoculation of upper respiratory microbiota by micro-aspiration, and clearance by respiratory endothelium cilia and cough.[33-35] Venkataraman et al. showed microbial composition of the healthy lung in adults was consistent with the neutral community model, i.e., bacterial dispersal from upper respiratory niches, particularly the oral cavity, explained lung microbial communities.[36] Interestingly, viable bacteria account for 61% of the recovered OTUs. In diseased lungs, however, selection seemed to shape bacterial communities, suggesting stable colonization. In healthy lungs, bronchioles and alveoli are covered with surfactant and its low content in nutrients impair bacterial growth.[37] In case of infection, the high protein content exudate resulting from inflammatory response serves as substrate for bacterial overgrowth. Studies have shown the composition of the microbiome of the lower airways in healthy adults to correlate with that of the upper airways, particularly the oropharynx, but to be much lower in abundance (from 10^5 bacteria per milliliter of nasopharyngeal fluid to 10^1 - 10^2/mL of BAL fluid).[38-41] Similar results have been obtained in children, except for a greater contribution of nasopharyngeal microbiota in lower airways' microbiota composition.[42] Segal et al. partially challenge these findings. In two cross-sectional studies with healthy adults, they determined two distinct pneumotypes based on bronchoalveolar lavage (BAL) samples, one enriched with bacteria from the upper respiratory tract and present in around 45% of participants, and the other enriched with background bacteria.[43,44] The first pneumotype was associated with increased pulmonary inflammation measured by exhaled nitric oxide and BAL cells count. This subclinical inflammation characterized by increased Th17 response might be beneficial for the host, as suggested by animal models of Staphylococcus aureus pneumonia.[45]

4. Respiratory microbiota and lower respiratory conditions

We systematically reviewed the literature to retrieve original papers describing associations between respiratory microbiota and lower respiratory tract infections (Box 1). We excluded HIV infected patients, as it was object of a recently published review.[3] A total of 25 articles were retained for this section, 13 in adults,[46-58] and 11 in children.[19,59-69] An overview of the studies characteristics is summarized in tables 2 (adults) and 3 (children).

4.1. Pneumonia and ventilator-associated pneumonia

Despite differences in study populations and samples, similar patterns have been observed in patients with pneumonia. In general, such patients display a more abundant microbiota with less diversity, richness and evenness, and these disturbances can be observed in upper and lower respiratory tract microbiota, particularly in adults. The microbiota of patients with pneumonia seems to be dominated by one taxon, showing a higher relative abundance of *Streptococcus*, *Haemophilus* or *Moraxella*. Dominance by *Streptococcus pneumoniae*, *Burkholderia*, *Bacillales*, and to a lesser extent, *Pseudomonadales* was particularly correlated with decreased diversity.[58,68] In studies with repeated sampling, the dominance of one taxon was maintained over time, and its relative abundance increased throughout the pneumonia episode, particularly if the patient was intubated.[51] Intubation was consistently a risk factor for bacterial disturbance in the lower respiratory tract (low diversity and evenness) supporting the hypothesis of a lung microbiota resulting from the equilibrium between microaspirations from the upper respiratory tract and clearance mechanisms.[34,51,58,63] Antibiotic administration seemed to have less effect on bacterial diversity than intubation,[58] and this effect was transitory, with recovery of a similar oropharyngeal microbiota a few days after antibiotic treatment compared to the microbiota before antibiotic administration.[52] However, most of the studies did not assess this effect, probably due to difficulties in assuming bacterial viability from DNA based measures.[70,71] In an early study assessing the effect of antibiotics in seven intubated adults colonized by *Pseudomonas aeruginosa*, Shannon's diversity index significantly fell from 1.48 to 0.59 after a few days of antibiotic treatment, with *P. aeruginosa* coming to dominate the microbiome in most patients despite susceptibility in vitro to the administered antibiotics.[72] No taxon was consistently associated with health, although some associations were found. *Prevotella* dominant oropharyngeal microbiota was more frequent in healthy adults than in adults with pneumonia, but more frequent in older children with severe pneumonia than in non-severe pneumonia cases.[46,66] *Veillonella* and *Dolosigranulum* were frequently sequenced in healthy individuals or patients with less severe pneumonia in upper respiratory tract samples, the latter particularly in young children.[46,66,67,73] Interestingly, despite *Moraxella* being associated with pneumonia, *Moraxella lacunata* was significantly more frequent in pediatric viral vs. non-viral pneumonia, and more frequent in controls than in all-cause pneumonia, highlighting the necessary caution in establishing associations based on phylum or genera-level taxonomics.[67]

Interesting inconsistencies merit further examination. In a study in adults with community-acquired pneumonia and healthy controls, oropharyngeal microbiota dominated by *Lactobacillales* (predominantly *Streptococcus spp.*) was associated with

pneumonia.[46] This result was partly consistent with a pediatric study assessing the association between upper and lower respiratory tract microbiota and pneumonia severity.[66] Presence of *Lactobacillales* was associated with longer hospital stay in children older than five years of age, but with a less severe pneumonia in under-five children underscoring the importance of age-related host-microbe interactions and immune system dynamics. Two studies did not support the association between low microbiota diversity and disease. Toma *et al*. compared the microbiota of endotracheal aspirates from intubated adults with possible ventilator-associated pneumonia and found no differences in diversity when comparing low and high-risk groups as per the Clinical Pneumonia Infection Score (CPIS).[56] However, there were no significant differences between groups in most of the items included in the CPIS, and the authors did not report clinical outcomes at the end of the admission, raising concerns of misclassification. Wang *et al*. compared the microbiota of bronchoalveolar lavage fluid in children with pneumococcal and *Mycoplasma* pneumonia with controls, and reported that controls had the lowest microbial diversity.[68] This could be evidence of age-dependent differences in health and disease, although caution is warranted at this stage of microbiome research. Noteworthy, controls (n=11) were younger than cases and had tracheomalacia, which may play a role in impaired clearance of the lower respiratory tract in individuals from an age group with a respiratory microbiota yet to be fully developed.

4.2. Bronchiolitis and asthma

Recurrent wheezing and asthma in childhood are strongly associated with having suffered a bronchiolitis episode during the first year of life, particularly when caused by respiratory syncytial virus (RSV) or human rhinovirus (HRV). We retrieved six original papers assessing the respiratory microbiota in infants with bronchiolitis, and one in children and adolescents with asthma, with all of them using upper airways samples.[19,59-62,64,69] The article on asthma was included because it focused on the infectious component of recurrent wheezing pathophysiology. Despite a smaller variability in study population and in sampling sites than in the aforementioned pneumonia studies, findings in this condition are discordant. While there is some degree of consensus in describing a decreased evenness in both asthma and bronchiolitis, discrepancies arise on which phylum or genus dominates the microbiota. Most studies associate *Proteobacteria* (phylum) and *Moraxella* or *Haemophilus* (genera) dominant taxa in upper respiratory microbiota with asthma, bronchiolitis or severity of bronchiolitis.[19,59,62,69] On the other hand, in a case-control study with infants with bronchiolitis and healthy controls, Hasegawa et al. described four distinct profiles of upper respiratory microbiota, each of them dominated by a taxon, with infants with the *Moraxella* dominant profile having the lowest risk of having bronchiolitis, and the *Staphylococcus* dominant profile presenting an odds ratio of 5 for bronchiolitis.[60] Interestingly, *Dolosigranulum* dominant profile also presented a very low risk of bronchiolitis, consistent with the findings we described above in the section dedicated to pneumonia. In another large case-series published by the same author assessing the association of microbiota and bronchiolitis severity, *Moraxella* dominant profiles presented again the lowest risk of a negative outcome (defined as admission to the intensive care unit).[61] In this series, the profile with highest risk of negative outcome was the *Haemophilus* dominant one, consistent with most of the studies. Seasonal or geographical effects do not explain these discrepancies as most of the studies were

conducted in the US in winter and merit further investigation. Four studies reported data on viral etiology and suggested virus-bacteria interactions in the pathogenesis of bronchiolitis and future recurrent wheezing, particularly between *Proteobacteria* and RSV.[19,62,69] The chronology of the interaction, i.e., whether a viral infection alters the epithelium environment allowing for a greater bacterial adherence or a colonizing bacteria predisposes to viral infection, is still unclear, although there is some evidence supporting the former at least in case of human rhinovirus infection (hRV). In a prospective study with weekly nasal samples collected from children aged four to twelve years old, detection of hRV increased the risk of concurrent or subsequent detection of pathogenic bacteria (either *S. pneumoniae*, *H. influenzae* or *M. catharralis*), whereas the opposite was not observed.[74] In a randomized controlled trial in infants with RSV-bronchiolitis admitted to hospital, Zhou et al. assessed the effect of a 14-days course of azithromycin (AZM) on future recurrent wheezing, reporting a 50% reduction over the following 12 months.[69] Nasal lavages samples were taken before and after the treatment showing a significantly higher reduction in *Moraxella* relative abundance in the treatment group. Cautiously, the authors recognized difficulties unfolding the direct anti-inflammatory effect of AZM on respiratory airways and the indirect effect through *Moraxella* depletion on the recurrent wheezing reduction. On the other hand, Mansbach et al. found that in infants hospitalized with severe bronchiolitis, RSV mono-infection was associated with high relative abundance of *Firmicutes* (*Streptococcus*) and low abundance of *Proteobacteria* (*Moraxella* and *Haemophilus*), with an inverse association in HRV mono-infection.[64] To note, this case-series is the same as the one described by Hasegawa et al.

4.3. Bronchiolitis *obliterans* syndrome (BOS)

Lung transplant recipients face a higher risk of acute and chronic rejection of the allograft than other solid organ recipients, with BOS being the leading cause of death after the first year.[75] Although epidemiologically less relevant than pneumonia and asthma, the dramatic outcome of BOS and the host-microbe interactions involved merit a closer attention. Traditionally, proper immunosuppression and aggressive antibiotic treatment, particularly against *Pseudomonas aeruginosa* have been the main pillars in BOS prevention and management.[76] Case-control studies using culture-independent techniques are providing notable insight that may change our perception of BOS pathogenesis.[47,57] Lung transplant patients harbor a more abundant and less diverse lower airways microbiota than non-transplant patients. Two main pneumotypes have been described according to genera dominance, one dominated by *Pseudomonas* and the other dominated by *Veillonella*, *Prevotella* or *Streptococcus*. These pneumotypes seem to be mutually exclusive, and although *Pseudomonas* abundance is associated with low diversity, which is in turn associated with higher BAL neutrophilia and bacterial DNA burden, assigning a healthy or unhealthy label to these pneumotypes is not straightforward. Dickson et al. assessed the association between BAL microbiota and BOS in 33 adults with lung transplant, and identified an uncommon *Pseudomonas* species, *Pseudomonas fluorescens*.[47] Almost all the patients with *P. aeruginosa* had symptoms associated with BOS, whereas all the patients with *P. fluorescens* were symptom-free. A case-control study with 57 lung transplant recipients and 8 controls conducted by Willner et al. yielded results requiring a close attention.[57] Half of the lung transplant recipients had cystic fibrosis (CF), with all of them being colonized by *P. aeruginosa* prior to transplantation. In this group,

recolonization by *P. aeruginosa,* usually from upper airways reservoirs, was negatively associated with BOS (OR 0.25; 95% confidence interval 0.09-0.65), whereas presence of newly acquired *P. aeruginosa* was associated with developing BOS in the non-CF subgroup. These results might partly be explained by the phenotypic changes suffered by *P. aeruginosa*, transitioning from an aggressive form with high expression of virulence factors when acquired *de novo* to a more passive phenotype after long-term colonization showing less invasiveness and thus eliciting immunogenic response.[77] The authors conclude the reestablishment of pre-transplant populations in the allograft appear to protect against BOS, which supports the hypothesis of immunogenic tolerance in airways inflammation.

4.4. Others

This section includes a miscellanea of lower respiratory tract conditions in adults, from non-transplanted cystic fibrosis and non-CF bronchiectasis to interstitial lung disease and idiopathic pulmonary fibrosis, all of them directly or indirectly associated with infection.[48,49,53,54] Three studies assessed the association between respiratory microbiota and lung function, measured by spirometry, and found a correlation between microbiota diversity and forced expiratory volume in 1 second (FEV1).[48,53,54] In one of these studies, however, the lung function deterioration seemed to be driven more by bacterial abundance than microbiota diversity or composition.[53] Patients with idiopathic pulmonary fibrosis had slightly lower diversity than controls (Shannon index 3.8 vs. 4.1) and similar microbiota composition with *Streptococcus*, *Prevotella* and *Veillonella* accounting for 50% of reads in both groups, but significantly higher abundance than controls. These findings were also reported in a sub-group analysis comparing progressors and non-progressors among cases. To note, controls included patients with moderate COPD, which is associated with lower microbiota diversity.[78] On the other hand, Garzoni et al. compared the microbiota composition and diversity in upper and lower respiratory tract samples from 24 patients with interstitial lung disease or *Pneumocystis jirovecii* pneumonia and 9 healthy controls, and found no differences in richness, diversity and presence of commensals such as *Prevotella* or *Acidaminococcaceae* suggesting no effect of respiratory microbiota in the disease process.[49] The small sample size and the variety of diagnoses in the disease group could arise concerns about the internal validity of this study, but if confirmed, these negative results could help researchers in narrowing the pathophysiology of host-microbe interactions in lung disease.

5. Conclusion

Despite the variability of study populations, samples and methods used in the original papers included in this review, patterns in the effect of respiratory microbiota on lower respiratory tract infections and infection-related conditions start to arise, although a clear healthy respiratory microbiota is still to be defined. Including metagenomics and metabolomics data to metataxonomics evidence accumulated to date could shed some light to the field, although hypothesis-driven research should be strongly encouraged to minimize the risk of getting "lost in transcription".

6. Expert commentary

Interpreting and comparing respiratory microbiota data across studies is a difficult task. The main issue when studying the respiratory microbiota compared to the gut microbiota is the invasiveness of lung sampling. Some attempts to overcome this have been suggested, such as combining nasopharyngeal and oropharyngeal samples based on the relationship between these sites and the lower respiratory tract microbiota, and an interesting assessment of the lung microbiota through exhaled breath condensate fluid that showed a high correlation with bronchoalveolar lavage fluid in intubated patients[79], although this technique does not seem to be easily applicable to non-intubated patients. Besides the difference in samples, variability in sequencing techniques, sequenced regions, library preparation, bioinformatics tools and reference databases among others could yield different results. Kumar et al., for example, reported differences in taxonomic results when sequencing different variable regions of the 16SrRNA gene and suggested that V1 to V3, and V7 to V9 showed the greatest utility in classification.[80] On the other hand, Kelly et al. reported similar results when comparing the performance of two sequencing platforms (Illumina MiSeq and Roche/454) on the same samples.[51] To avert these issues, it is necessary to standardize existing techniques, to keep establishing associations between the microbiota from different sampled respiratory sites, and ideally, to create a unique genome reference database. In addition, molecular biology tools to assess viability of bacteria identified by sequencing methods should be applied when possible. This would help reduce the intricacy of microbiota projects' results and establishing patterns, a key step to move from the observational nature of the microbiota studies to date to hypothesis driven and interventional projects.

Most of the studies assessing associations between the respiratory microbiota and disease are metataxonomic studies (i.e., based on 16SrRNA sequencing) with relatively small sample sizes. Even with statistical corrective measures to account for multiple comparisons, spurious associations cannot completely be ruled out. Multiplicity of comparisons is an intrinsic issue in microbiota research because of the myriad of bacterial species co-existing in the human body, and the multiple characteristics we use to describe bacterial communities, i.e., evenness/dominance, richness, abundance and presence of specific taxa. However, there appears to be a certain consistency in microbiota studies where diversity is usually associated with health, and dominance of a single species usually associated with disease. Importantly, these could just be proxies of respectively presence and absence of a particular bacterial consortium creating a specific environment with protective or beneficial effect. This hypothesis is supported by some studies with animal models showing that resistance against *Clostridium difficile* colonization was not conferred by any single bacterial population, but rather by combinations of different commensals.[81]

Moreover, the dynamics and the effect over time of the respiratory microbiota have scarcely been studied raising reasonable doubts about the causal relationship between findings in microbiota composition and disease. Besides the cohort study conducted by Teo *et al*. and described in the "Asthma" section, another cohort study led by Santee *et al*. showed that dominance of either *Moraxella*, *Streptococcus* or *Haemophilus* in the nasopharynx of children aged 4 to 7 years was associated with more frequent

upper respiratory tract infections including sinusitis over a 1-year follow-up.[82] These findings should encourage researchers to conduct cohort studies in humans and interventional studies in animal models to validate the results from cross-sectional studies as a necessary step towards designing preventive measures against respiratory conditions based on the respiratory microbiota. Ideally, these potential novel measures should be applicable in developing countries, the world's region that suffers the highest burden of pediatric respiratory infections, and that is undergoing an epidemiological transition towards a double burden of disease with an important role of chronic respiratory conditions.

7. Five-years view

Currently, the dominance of host transcripts and metabolites in respiratory samples obscures the interpretation of costly transcriptome and metabolome studies. With decreasing costs of culture-independent techniques, particularly whole genome sequencing, innovative solutions to overcome interpretation issues of results from omic platforms, and full integration with other disciplines (e.g., immunology, bioinformatics, microbiology, epidemiology), the bacterial microbiota research is likely to become a true pan-microbiome field with a comprehensive understanding of bacterial, viral, fungal and host interactions. The development of biobanks are a good ally for this endeavor, insofar it will enable analyzing large numbers of stored samples once the costs of omic platforms decrease exponentially. This will constitute the basics towards interventional research where probiotics, prebiotics and nutrition will probably be joined by phages and extremely narrow spectrum antibiotics as key interventions to build a healthy respiratory microbiota to be used as a preventive and therapeutic measure. Taking into account environmental effects, population and individual diversity, cross-talk between microbial communities from different body sites, and the effects of the microbiota on different diseases and conditions, the microbiome research could become one of the pillars of personalized medicine.

Key issues

- The respiratory tract is a complex ecosystem with distinct bacterial communities present in every niche, including the lungs.
- The respiratory microbiota seems to have an effect on lower respiratory infections and infectious-related conditions, where host-microbe and microbe-microbe interactions are not yet completely understood.
- Although microbiota diversity is usually associated with health, it is unclear whether diversity may mask the presence of protective bacterial consortia. Moreover, differences in children and adults might be evidence of important interactions associated with immune system development.
- Prospective studies with serial samples to describe the respiratory microbiome acquisition and dynamics, and to determine long-term effects on respiratory conditions are needed to exploit the translational potential of the respiratory microbiome.

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Box 1. Search strategy and selection criteria

We searched PubMed (accessed on February 18th 2017) and the reference lists of selected papers to identify studies assessing the association between respiratory microbiota and lower respiratory infections, limited to humans, and with no language restriction. We limited the search to studies using culture-independent techniques to identify and classify bacterial populations in order to increase comparability. We used a combination of search terms as both free and MeSH terms when applicable, to optimize sensitivity: ((microbiome) OR (microbiota)) AND ((airway*) OR (respiratory tract) OR (nose) OR (nasal) OR (mouth) OR (oral) OR (pharyn*) OR (nasopharyn*) OR (oropharyn*) OR (lung) OR (pulmonar*)) AND ((pneumonia) OR (bronchiolitis) OR (LRTI) OR (lower respiratory tract infection) OR (ARTI) OR (acute respiratory tract infection) OR (ARI) OR (acute respiratory infection)). A total of 125 articles were obtained after excluding animal studies. We screened titles and abstracts, and we retained original papers within the scope of the review.

Table 1. Terminology frequently used in microbiome research

Terminology	
Microbiota	Collection of microorganisms present in a particular site
Microbiome	Collection of microorganisms in a particular site, their genes, and the surrounding environmental conditions
Mycobiome	The entire genomic content of fungi in a particular site
Virome	The entire genomic content of viruses in a particular site
Metataxonomics	High-throughput process to characterize the microbiota and to establish taxonomic associations, usually by 16S rRNA sequencing
Metagenomics	High-throughput process to characterize the pool of genes from members of a microbiota, usually by shotgun sequencing
Metatranscriptomics	High-throughput process to identify and quantify microbial genes that are expressed by the microbiota
Metabolomics	Study of products of cellular metabolism in a defined environment, including host's and microbe's metabolites
Next-generation sequencing	In contrast with Sanger sequencing, NGS is based on parallel sequencing of vast numbers of short reads
Whole-genome Shotgun sequencing	Method to characterize whole genomes by sequencing a large number of random small DNA fragments in parallel and assembling them into larger contigs
16S rRNA gene sequencing	Method to characterize bacterial populations by sequencing variable regions of the highly conserved bacterial 16S rRNA gene
Operational Taxonomic Unit	Clusters of similar sequence variants of the 16S rRNA gene used as a surrogate for taxa in metataxonomics studies. 97%

(OTU)	similarity is commonly used as cut-off for species surrogate
Abundance	Total number of bacterial individuals in a sample
Richness	Number of different species/OTUs present in a sample
Evenness	Measure of similarity in relative abundance / frequency distribution of OTUs within a community. Dominance of one taxa implies decreased evenness
Alpha Diversity	Measure of diversity within a sample, taking into account richness and/or evenness. Commonly used metrics of α-diversity include Chao1, Shannon and Simpson indexes
Chao 1 Index	Measure of observed and unobserved richness
Shannon Index	Measure of diversity combining richness and evenness. Higher values indicate higher diversity
Simpson Index	Measure of dominance (complimentary measure of evenness). Higher values indicate lower evenness
Beta Diversity	Measure of diversity between samples. High β-diversity implies large dissimilarities between samples

Table 2. Overview of study characteristics included in the systematic review (Adults)

Author, year	Study design	Sample size	Baseline condition	Outcome of interest	Respiratory sample	NGS (variable region)	Reference
de Steenhuijsen, 2016	Case-control	127 cases 278 controls	None	Pneumonia	OP swab	16S rRNA (V5-V7)	46
Kawanani, 2016	Cohort	42	None	MRSA pneumonia	BAL	16S rRNA	50
Kelly, 2016	Cohort	15	Intubated patients	VAP	OP swab BAL	16S rRNA (V1-V2)	51
Lu, 2015	Case-control	30 cases 60 controls	Cirrhosis	Pneumonia	OP swab	16S rRNA (V3)	52
Shankar, 2016	Cross-sectional	16	Lung transplant	Pneumonia	BAL	16S rRNA (V4)	55
Toma, 2014	Cross-sectional	44	Intubated patients	VAP	ET aspirate	16S rRNA V1-V9	56
Zakhakina, 2017	Cohort	35	Intubated patients	VAP	ET aspirate	16S rRNA	58
Willner, 2013	Cohort	57	Lung transplant	BOS	BAL	16S rRNA (V7-V8)	57
Dickson, 2014	Case-control	33 cases 26 controls	Lung transplant	BOS	BAL	16S rRNA (V3-V5)	47
Flight, 2015	Cross-sectional	59	Cystic fibrosis	Lung function	Sputum	16S rRNA	48
Garzoni, 2014	Case-control	18 cases 15 controls	Interstitial lung disease	NA	OP swab BAL	16S rRNA (V3-V5)	49
Molyneaux, 2014	Cohort	65	Idiopathic pulmonary fibrosis	Lung function	BAL	16S rRNA (V3-V5)	53
Rogers, 2013	Cross-sectional	41	Non-CF bronchiectasis	Lung function	BAL Sputum	16S rRNA	54

Table 3. Overview of study characteristics included in the systematic review (Children)

Author, year	Study design	Sample size	Baseline condition	Outcome of interest	Respiratory sample	NGS (variable region)	Reference
Lu, 2014	Cross-sectional	25	Intubated newborns	VAP	ET aspirate	16S rRNA (V3)	63
Pettigrew, 2016	Cohort	383	Pneumonia	Severity	Sputum NP swab OP swab	16S rRNA (V4)	66
Sakwinska, 2014	Case-control	50 cases 50 controls	None	Pneumonia	NP swab	16S rRNA (V1-V2)	67
Wang, 2016	Case-control	21 cases 11 controls	None	Pneumonia	BAL	16S rRNA (V3-V4)	68
Castro-Callar, 2015	Case-control	8 cases 6 controls	None	Asthma	Nasal brush	Whole genome shotgun	59
Hasegawa, 2016	Case-control	40 cases 110 controls	None	Bronchiolitis	Nasal swab	16S rRNA (V4)	60
Hasegawa, 2016	Cross-sectional	1005	Bronchiolitis	ICU admission	NP aspirate	16S rRNA (V4)	61
Hyde, 2014	Cross-sectional	100	Bronchiolitis	NA	NP aspirate	16S rRNA (V3-V5)	62
Mansbach, 2016	Cross-sectional	740	Bronchiolitis	NA	NP aspirate	16S rRNA (V4)	64
Teo, 2015	Cohort	234	High risk of atopy	Asthma LRI	NP aspirate	16S rRNA (V4)	19
Zhou, 2016	Cohort	39	Bronchiolitis	Recurrent wheezing	Nasal lavage	16S rRNA (V1-V3)	69

VAP: ventilator-associated pneumonia; ET: endotracheal; NP: nasopharyngeal; OP: oropharyngeal; BAL: bronchoalveolar lavage; ICU: intensive care unit; LRI: lower respiratory infection