



# A clinicians' review of the respiratory microbiome

Christina D. Campbell <sup>1</sup>, Clea Barnett<sup>2</sup> and Imran Sulaiman <sup>1,3</sup>

<sup>1</sup>Royal College of Surgeons in Ireland, Dept of Respiratory Medicine, Dublin, Ireland. <sup>2</sup>New York University Grossman School of Medicine, Division of Pulmonary and Critical Care Medicine, New York, NY, USA. <sup>3</sup>Beaumont Hospital, Dept of Respiratory Medicine, Dublin, Ireland.

Corresponding author: Christina D. Campbell ([christinadcampbell@rcsi.com](mailto:christinadcampbell@rcsi.com))



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**The respiratory microbiome encompasses bacterial, fungal and viral communities. In health, it is a dynamic structure and dysbiotic in disease. Dysbiosis can be related to disease severity and may be utilised to predict patients at clinical risk.** <https://bit.ly/3pNSgnA>

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## Abstract

The respiratory microbiome and its impact in health and disease is now well characterised. With the development of next-generation sequencing and the use of other techniques such as metabolomics, the functional impact of microorganisms in different host environments can be elucidated. It is now clear that the respiratory microbiome plays an important role in respiratory disease. In some diseases, such as bronchiectasis, examination of the microbiome can even be used to identify patients at higher risk of poor outcomes. Furthermore, the microbiome can aid in phenotyping. Finally, development of multi-omic analysis has revealed interactions between the host and microbiome in some conditions. This review, although not exhaustive, aims to outline how the microbiome is investigated, the healthy respiratory microbiome and its role in respiratory disease.

## Educational aims

- To define the respiratory microbiome and describe its analysis.
- To outline the respiratory microbiome in health and disease.
- To describe future directions for microbiome research.

## Introduction

The respiratory microbiome and its impact on health and disease is increasingly well characterised. Further work on microbial function in the lower airways is ongoing. With the advent of culture-independent techniques and next-generation sequencing (NGS), we have a broader understanding of the microbiome, as well as investigating its functional capacity and therefore, potential direct impact on the host in health and disease. In this review, we will discuss how the microbiome is investigated, the healthy lung microbiome and its role in respiratory disease. In the past, traditional culture-based techniques were used to assess the microbial universe of different environments, however, these techniques are selective and don't provide the full picture [1]. NGS has opened a new door to understanding the interplay between microorganisms and humans in different microenvironments.

## Sources and search strategy

References used for this review were identified from PubMed Central and MEDLINE searches. Search teams used included "microbiome", "microbiota", "metabolome", "virome", "mycobiome", "metagenomic", in conjunction with specific disorders, including "lung", "respiratory", "oropharyngeal", "upper respiratory tract", "asthma", "COPD/chronic obstructive pulmonary disease", "interstitial lung disease", "IPF/idiopathic pulmonary fibrosis", "cystic fibrosis", "bronchiectasis", "lung cancer". Publications in high-impact journals, specifically targeting the lower airways were prioritised where possible.



### **What is the microbiome?**

Two commonly used terms from culture-independent techniques include the microbiome and microbiota. In practice they are used interchangeably but do have differences. The microbiome is defined as the combined genomes (omic) of all the microorganisms inhabiting a specific environment [2], such as the gut, genitourinary tract or even the lung. That is to include the microorganisms and their genes. Whereas the microbiota refers to the community of microorganisms themselves [3]. Although most research has focused on bacteria at present, NGS allows us to explore other microorganisms such as viruses, fungi and archaea.

### **How is the microbiome assessed?**

#### ***16S rRNA gene sequencing***

During the late 1970s, sequencing of a small subunit ribosomal gene, referred to as 16S rRNA was pioneered to assess bacterial phylogeny [4, 5]. This gene is of particular use, as it is a conserved component of the transcriptional apparatus of all DNA-based lifeforms [6]. It consists of nine constant regions and nine hypervariable regions (V1–V9) [7]. It is the examination of these hypervariable regions that allows for discrimination between different bacteria, generally one or two hypervariable regions are selected. NGS of 16S rRNA *via* gene amplicon sequencing allows us to analyse the bacterial composition of many samples at the same time and at low cost. The sequences amplified can then be mapped to a database, leading to quantitative estimates of bacteria present in a sample.

Ecologically, microorganisms are classified taxonomically into Kingdom, Class, Order, Phylum, Genus and Species. However, amplicon sequences often do not correspond to the classically determined taxonomies. As a consequence, clusters of similar sequences are grouped into operational taxonomic units (OTUs). The main disadvantage of the use of OTUs is that errors in a reference database will be reflected in the analysis and novel taxa cannot be detected.

Amplicon sequence variant (ASV) is a newer approach, which determines exactly what sequences were detected and their frequencies. Comparison of similar frequencies determines the probability a particular read is not due to a sequencing error. As ASVs are exact sequences, they can be compared to reference databases at a higher resolution.

#### ***Fungal internal transcribed spacer sequencing***

NGS of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA (rRNA) is utilised to assess fungal phylogeny, and from that assessment of the mycobiome [8]. Due to the read length limitations of some sequencing platforms, at times only parts of the ITS region is used, *i.e.* ITS1 or ITS2, similar to selection of variable regions in 16S sequencing. This selection should be made with care, as some primers can bias toward different phyla [8].

#### ***Whole genome sequencing (metagenomics)***

16S rRNA and ITS sequencing describe the abundance of bacteria and fungi present in a specific environment. The advancement of NGS has led to the development of whole genome sequencing, also referred to as metagenomics (meta referring to microorganisms as opposed to human genomics) [9]. With this new technology, we can sequence for the abundance of bacteria as well as viruses (the virome), fungi (the mycobiome) and archaea all at once. Functional capacity of the community can then be deduced from the presence of particular genes; however, the expression patterns of particular genes cannot be inferred with this technique [10].

#### ***RNA metatranscriptomics***

Metagenomics provides information on functional potential of microorganisms, while RNA metatranscriptome sequencing allows us to truly interrogate what microorganisms are actively transcribing. The metatranscriptome is a measure of the total mRNA in a sample, providing a representation of active gene expression in a sample at that moment in time [10].

#### ***Resistome***

With the discovery of antibiotics, emergence and dissemination of resistance followed soon after. This resistance is mediated mainly by antibiotic resistance genes (ARGs) produced by antibiotic producing and non-pathogenic bacteria [11]. Metagenomics and metatranscriptomics, using NGS, has been applied to the human microbiome to assess the resistome. The resistome of the human microbiome is considered an important reservoir for dissemination of clinical ARGs. Analysis of the resistome of the respiratory microbiota has revealed a core respiratory resistome, even in healthy airways [12].

### Ecological terminology

The majority of terms used to describe the human microbiome are derived from ecology. Commonly used metrics in microbiome analysis include alpha diversity and beta diversity. Alpha diversity describes the ecological diversity within a sample. This can refer to the richness (number of species), or evenness (distribution of species). There are many metrics used to calculate alpha diversity, the most simple being a count of all the different OTUs present. The most commonly used is the Shannon diversity index, an account of abundance and evenness of species present. Beta diversity is a measure of the similarity or dissimilarity of two communities. Again, many indices are available to describe beta diversity. Bray–Curtis distance is a statistical tool which quantifies the compositional dissimilarity between two samples, giving a value ranging from 0 to 1, 0 meaning the groups share all species and 1 meaning that they share none. Weighted UniFrac distance estimates differences between samples based on phylogenetic distance, while also incorporating abundance values. The use of phylogenetic distance aims to reduce the contribution of rare species. Another commonly used term is dysbiosis, this refers to a change in the composition of the microorganisms in the microbiome, which results in a reduction in their ability to function normally.

### Metabolomics and proteomics

Metabolomics is a developing field in microbiome analyses aimed at understanding the by-products of organisms. Metabolomic analysis, through mass spectrometry, can identify and quantify the metabolites of any given sample, such as fructose–rhamnose and cellobiose, carbohydrates synthesised by bacteria which can represent active bacterial metabolism [13]. The metabolome can reflect the health of an environment or show evidence of dysbiosis [14]. Assessment of metabolites can identify metabolically active organisms which produce metabolically active metabolites with immunomodulatory capabilities, such as short-chain fatty acids [13]. Proteomics is a similar technique using mass spectrometry to study the expression, structure, function and interactions of a set of proteins produced by the host and microbiome (table 1).

Although providing a vast amount of information about the microbial composition of different environments, there are some limitations to NGS. Most bacteria are only identified at a genus level, and furthermore sequencing cannot always distinguish between differing strains. Contamination can occur at the time of sampling, during DNA extraction or in the course of PCR amplification. Measures can be undertaken to minimise the influence of contamination, for example, concern over contamination from bronchoscopes can be overcome with simultaneous sequencing of background bronchoscopy and bronchoalveolar lavage (BAL) samples, allowing identification of any taxa that are possible contaminants [16]. To overcome contamination from DNA extraction kits and reagents, concurrent sequencing of negative controls can be used [17]. In addition, statistical packages, such as the *decontam* package in R, can also be used to help identify contaminants. It is important to understand there are areas of the human body which have low amounts of bacteria, such as the lung, joints and breasts. The paucity of organisms can be a further challenge to NGS.

The combination of all the above techniques is referred to as multi-omics, and can be applied to the respiratory microbiome to describe taxonomic composition, functional and metabolic activity of the microbiome. With these techniques, the modern study of the human microbiome came to the forefront in 2007, with the inception of the Human Microbiome Project [18]. This was designed to characterise the human microbiome in the nasal passages, oral cavity, skin, gastrointestinal tract and urogenital tract. Through this integrated analysis it is now clear the gut microbiome has a role in modulating systemic

**TABLE 1** Summary of definitions

<b>Microbiome</b>	The combined genomes of all the microorganisms inhabiting a particular environment
<b>Microbiota</b>	Community of microorganisms in an environment
<b>Metagenomics</b>	The study of the whole genome of a community of microorganisms, allowing assessment of the functional potential of the microorganisms
<b>RNA metatranscriptomics</b>	The measurement of the total mRNA in a community, to allow representation of active gene expression at that time
<b>Resistome</b>	Collection of antibiotic resistance genes in a community
<b>Metabolomics</b>	Assessment of the metabolites of a given sample, allowing identification of metabolically active organisms
<b>Proteomics</b>	Assessment of proteins produced by the host and microbiota
<b>Alpha diversity</b>	The diversity of a sample, including the richness (number of species) or evenness (distribution of species)
<b>Beta diversity</b>	The measure of the similarity or dissimilarity of two communities

immune tone and drug effectiveness [19, 20]. Furthermore, the role of the gut microbiome in disease is becoming apparent, with recognition of the gut–brain axis in mental health disorders, and its role in metabolic conditions, including obesity and type two diabetes among others [20]. Currently, most studies investigating the respiratory microbiome are observational and the functional potential remains under-investigated. Here we will review some of the literature using the above techniques in evaluating the respiratory microbiome in both health and in specific respiratory diseases.

### **The development of the respiratory microbiome**

The respiratory microbiome is established at birth and appears essential for normal lung development [21]. It then develops rapidly in the first few weeks of life. Many factors influence the development including breastfeeding [22], antibiotic use, vaccination, siblings, smoke exposure, environment and childhood infections. The childhood microbiome can also affect the development of respiratory diseases in later life, including asthma [23].

### **The respiratory microbiome in health**

The respiratory microbiome exists along a gradient, with microbial diversity highest in the upper respiratory tract and decreasing towards the lower respiratory tract [24]. The upper respiratory tract provides a variety of niches for bacterial colonisation and the microbiome varies by location [25].

The upper airway microbiome is complex, as each surface presents a different ecological niche, each with unique conditions suitable for variable organisms. Hence, the microbiome will vary between the hard and soft tissue, saliva, tongue and plaque [26]. Nevertheless, in general the healthy upper airway microbiome is dominated by *Streptococci*, followed by *Neisseria*, *Prevotella*, *Rothia* and *Haemophilus*. Of note, anaerobes are uniquely represented in plaque specimens [27].

The upper airway virome is rich and complex, including several phages of the *Siphoviridae*, *Myoviridae* and *Podoviridae* families, which are directed against the most abundant bacterial species in the oral microbiome. Additionally, viruses of the *Herpesviridae* family are abundant [27]. The upper airway mycobiome is mainly *Candida* and *Saccharomycetales* [27].

Sputum is often used as a surrogate for the lower airway microbiome. However, it is important to recognise that expectorated sputum exists as a combination of secretions in the lower respiratory tract and upper airways, including the oral microbiome. Direct comparison between sputum and BAL has shown that sputum contains a bacterial load comparable to that of the upper airways, with significant differences in beta diversity between all samples. Sputum samples are most similar to supraglottic samples, indicating that sputum is not an accurate representative of the lower airway microbiome [28]. Sputum may be a representative sample in diseases with high sputum volume, such as cystic fibrosis (CF), but investigators should be aware of the drawbacks.

The lower respiratory tract has significantly reduced bacterial concentration and differing diversity compared with the upper airway. The microbiome is characterised by Bacteroidetes, Proteobacteria, Firmicutes and at genus level, *Streptococcus*, *Prevotella* and *Veillonella* [29]. *Anelloviridae* form the majority of the lower airway virome with herpes viruses and human papillomavirus [30]. The mycobiome consists of *Ceriporia lacerate*, *Saccharomyces cerevisiae* and *Penicillium brevicompactum* [31].

It is important to appreciate that the healthy respiratory microbiome is transient, with microbes moving into the lung by inhalation, micro-aspiration and direct mucosal spread from the upper airway, and then being removed by the mucociliary system and innate immune system. Next, we will review the changes in the respiratory microbiome in several common respiratory diseases (table 2).

### **The respiratory microbiome in disease**

#### ***Asthma***

Asthma is a heterogeneous condition characterised by airway inflammation and hyperreactivity which, at an immunological level, can be divided into T-helper cell (Th)2 high or Th2 low phenotypes. A long-standing hypothesis in the pathogenesis of asthma relates to hygiene and exposure to irritants. Indeed, further exposure to irritants, such as pollen may increase symptoms in patients with asthma. One area of interest is understanding the respiratory microbiome in asthma and how it relates to different phenotypes and symptom control.

Overall asthmatic patients display a higher bacterial burden compared with healthy controls when assessed via bronchial epithelial brushings [32]. There is a loss of low-level commensal organisms on oropharyngeal

TABLE 2 Summary of the respiratory microbiome in health and disease

Respiratory microbiome	Micobiome	Virome	Mycobiome
<b>In health</b>	Bacteroidetes Proteobacteria Firmicutes Genus: <i>Streptococcus</i> , <i>Prevotella</i> , <i>Veillonella</i>	<i>Anelloviridae</i> Human herpes viruses Human papillomavirus	<i>Ceriporia lacerate</i> <i>Saccharomyces cerevisiae</i> <i>Penicillium brevicompactum</i>
<b>Asthma</b>	Proteobacteria <i>Streptococcus</i> Reduction in <i>Prevotella</i> and <i>Veillonella</i>	Cytomegalovirus Epstein–Barr virus	<i>Psathyrella candolleana</i> <i>Termitomyces clypeatus</i> <i>Grifola sordulenta</i> <i>Malassezia pachydermatis</i>
<b>COPD</b>	Proteobacteria <i>Haemophilus</i> <i>Streptococcus</i>	Picornaviruses Influenza Parainfluenza Respiratory syncytial viruses Adenoviruses	<i>Alternaria</i> <i>Aspergillus</i> <i>Cladosporium</i> <i>Cryptococcus</i> <i>Mycosphaerella</i> <i>Penicillium</i> <i>Trametes</i> <i>Wickerhamomyces</i>
<b>Bronchiectasis</b>	<i>Pseudomonas</i> <i>Haemophilus</i> <i>Veillonella</i> <i>Prevotella</i>	<i>Siphoviridae</i> <i>Caudovirales</i> <i>Myoviridae</i> <i>Phycodnaviridae</i>	<i>Aspergillus</i> <i>Cryptococcus</i> <i>Clavispora</i>
<b>CF</b>	<i>Pseudomonas</i> <i>Staphylococcus</i> <i>Stenotrophomonas</i> <i>Burkholderia</i>	Influenza Respiratory syncytial virus	<i>Aspergillus</i> <i>Candida</i>
<b>IPF</b>	<i>Haemophilus</i> <i>Neisseria</i> <i>Streptococcus</i>	Influenza A Human herpesvirus	Not yet established
<b>Lung cancer</b>	<i>Veillonella</i> <i>Streptococcus</i> <i>Prevotella</i>	Not yet established	Not yet established

swabs [33], with an expansion in Proteobacteria [29, 32, 33], and *Streptococcus* in severe asthma [29, 32, 34] with a concurrent reduction in the abundance of *Prevotella* and *Veillonella* [35]. These findings are independent of inhaled corticosteroid use, with a similar microbial diversity reported in mild asthmatic patients [36]. In fact, Proteobacteria is one of the most prevalent bacterial phyla found in asthma patients, and is associated with worsening symptomology, steroid responsiveness and Th17 and interleukin (IL)-17 driven inflammation [34]. All which promote neutrophil recruitment and neutrophilic airway inflammation [34]. Interestingly, individuals with Th2 inflammation display a higher bacterial burden when compared to Th2-low asthma [36]. Furthermore, expansion in Proteobacteria, sphingomonadaceae and oxalobacteraceae on bronchial brushings are associated with bronchial hyperresponsiveness [32]. Of note, sphingomonadaceae have cell membrane glycosphingolipids, which can activate invariant natural killer T-cells, inducing IL-4 and IL-13, demonstrating a possible relationship between microbiota and local immunological tone.

In asthma there can also be an expansion in potentially pathogenic bacteria which are carried in the airway microbiome of the healthy population [33]. This suggests a diverse microbial community in health may protect against asthma, by inhibition of potential pathogens; the loss of commensal organisms leads to a loss of local or systemic immune tone which then signals to downregulate immune responses coupled with intermittent mucosal damage by potentially pathogenic bacteria, which are in excess in asthmatic airways.

The mainstay of treatment in asthma, particularly Th2-high asthma, is inhaled corticosteroids. However, some patients have been found to be corticosteroid resistant. Treating these patients can be quite challenging. The respiratory microbiome might provide some new therapeutic options. In corticosteroid-resistant asthma prior studies have found an abundance of Gram-negative lipopolysaccharide (LPS) producing bacteria in BAL with concurrent elevation of IL-8 and LPS, suggesting microbial stimulation [34]. Continuous airway stimulation by LPS has been shown to suppress Th2 activation [36]. *In vitro*, exposure to *Haemophilus parainfluenzae* leads to high levels of p38 MAPK activation and

reduced cellular response to corticosteroids in peripheral blood monocytes and BAL macrophages. Additionally, lack of response of pulmonary macrophages to corticosteroids leads to persistent airway inflammation in corticosteroid-resistant asthma [37].

Patients with uncontrolled asthma tend to have frequent exacerbations, with worsening of symptoms (such as shortness of breath and wheeze) frequently requiring systemic corticosteroids and sometimes antibiotics. While exacerbations are associated with *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Klebsiella* species [38], *H. influenzae* is the most common pathogenic bacteria isolated in induced sputum from asthmatic patients [39]. Interestingly, early childhood colonisation by potential pathogens, *S. pneumoniae*, *M. catarrhalis* or *H. influenzae* isolated from hypopharyngeal aspirates, confers an increased risk for recurrent wheeze or asthma in childhood [23]. The bacterial burden of *H. influenzae* in induced sputum has been shown to predict treatment response to azithromycin [40], a commonly used antibiotic in respiratory diseases. In an interesting study examining the effects of azithromycin on the respiratory microbiome, following treatment there is a reduction in bacterial richness and altered airway microbiota, leading to *Enterococcus* becoming the dominant microorganism in asthmatic BAL [41]. While *Pseudomonas*, *Haemophilus* and *Staphylococcus* decrease in abundance in the BAL [41], all potentially pathogenic genera. Interestingly, increasing relative abundance of the Firmicutes phylum on bronchial brushing is negatively correlated with symptoms [34], indicating both an antibiotic and immunomodulatory effect of azithromycin. Thus, specific pathogenic bacteria may influence the response to pharmacotherapy and colonisation by pathogenic bacteria may be potential targets for intervention.

Severe asthma is often linked with fungal sensitisation. Analysis of induced sputum from asthmatic patients has revealed asthmatic individuals have a distinct mycobiome to that of healthy controls [42]. Of interest, three of the four commonest species identified were from the macromycetes (mushroom) group: *Psathyrella candolleana*, *Termitomyces clypeatus* and *Grifola sordulenta*; the remaining species, *Malassezia pachydermatis*, is a yeast associated with atopic dermatitis. Asthma is associated with damp environments, yet this is the first reported association between macromycetes and asthma [42]. Additionally, analysis of the BAL mycobiome has shown that inhaled corticosteroid treatment is associated with an increased fungal burden of *Aspergillus* [43].

Viruses also play a key role in the development of asthma and subsequent exacerbations, notably rhinoviruses, influenza and respiratory syncytial viruses [44]. The respiratory virome in asthma patients is distinct to that of healthy controls, with an abundance of Herpes viruses, predominantly Cytomegalovirus and Epstein–Barr virus seen in sputum. Notably, their abundance was associated with more severe disease, increased symptomology and decreased lung function [45].

Individuals with asthma are frequently prescribed antimicrobial therapy both acutely for exacerbations and chronically for symptom control. Given the global burden of antimicrobial resistance, selection pressure leading to antibiotic resistance is a key concern. Analysis of the resistome in the sputum of individuals with asthma has identified a core macrolide resistome, similar to that seen in healthy airways and with less resistance genes in comparison to COPD or bronchiectasis [12]. Notably, long-term exposure to azithromycin increases the abundance of several macrolide resistance genes, and has been associated with clinical resistance to *H. influenzae* [46].

It is now apparent that in asthma there are significant alterations to the respiratory microbiome. This dysbiosis has been linked to specific phenotypes and exacerbations. The functional effects of the respiratory microbiome and potential areas for interventions, as well as longitudinal studies are required to fully elucidate the role of the microbiome and develop therapeutic targets.

### COPD

COPD is characterised by airway inflammation, chronic bronchitis, emphysema and fixed airflow obstruction usually associated with cigarette smoke exposure. Chronic symptoms include shortness of breath, wheeze and a daily cough. Patients are also prone to exacerbations of COPD with worsening of symptoms usually precipitated by a bacterial or viral infection. The respiratory microbiome has been studied both to understand the pathogenesis of COPD, but also to understand disease progression.

Not surprisingly, with chronic airways inflammation the respiratory microbiome differs in COPD when compared with healthy controls, with a predominance of Proteobacteria and *Streptococcus* in COPD [47–49]. With disease progression and parenchymal destruction (notably so in emphysema), there is a decline in microbial diversity with a further expansion of Proteobacteria, particularly *Haemophilus* with a reduction in Firmicutes [47].

Reductions in alpha diversity have been associated with poorer lung function, more severe disease and a reduction in survival. These changes produce a measurable host response in lung tissues, as demonstrated by upregulation of host interferon and proinflammatory signalling pathways, neutrophilic infiltration and formation of neutrophil extracellular traps (NETs) associated with *Haemophilus* and *Moraxella* [47, 50, 51]. This suggests that a persistent low-level inflammatory immune response associated with the progression of COPD [52] may be driven by bacteria which expand on diminishing bronchiolar and alveolar surfaces [53]. In stable COPD, the sputum microbiome has been shown to be related to long-term survival with a clear relationship between the microbiome and clinical phenotypes. For example, a Proteobacteria-dominated microbiome is associated with chronic bronchitis, eosinopenia, neutrophilic inflammation, poorer lung function and decreased survival [50, 54]. While a *Streptococcus*-dominant microbiome is associated with raised peripheral blood eosinophils and absence of chronic bronchitis.

As with asthma, inhaled therapy is the cornerstone of treatment in COPD. However, as with asthma, long-term therapy can also induce alterations in the microbiome; inhaled corticosteroids lead to an increase in Proteobacteria (*Haemophilus*) [55] and expansion of *Streptococcus* [56], and long-acting  $\beta$ -agonist (LABA) monotherapy leads to an increase in Bacteroidetes (*Prevotella*) [57].

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification of COPD includes evaluating how frequently patients have exacerbations, as this has been shown to be associated with prognosis and helps direct treatment. Frequent exacerbators (GOLD C or D) have lower biodiversity, even during periods of stability, with an increase in *Streptococcus*, *Haemophilus* and *Moraxella* [58]. During exacerbations, there is a further reduction in biodiversity with a dominance of pathogenic taxa [48, 50, 59, 60]. It remains unclear whether these profiles are as a result of disease or intervention; meaning treatment during exacerbations, predominately steroids and antibiotics, further alter the respiratory microbiome [50]. It is likely a combination of the two.

The respiratory mycobiome in COPD, assessed by sputum, exhibits greater diversity in stable COPD compared with healthy controls [61]. Notably, the mycobiome composition is unchanged in differing GOLD stages or lung function. However, it does vary by exacerbation frequency. Two distinct patient clusters were identified based on mycobiome signatures: one cluster characterised by increased symptoms and predominance of *Saccharomyces* and another cluster characterised by exacerbations, increased mortality and *Aspergillus*, *Curvularia* and *Penicillium*. This cluster also exhibits a systematic immune response to fungi [61].

Viral infections have long been a major driver of acute exacerbations in COPD; however, the virome is less well studied. There is evidence that viral infections lead to alterations in the bacterial microbiome, with consequent bacterial overgrowth and infection [62]. The predominant pathogens detected during exacerbations are picornaviruses, followed by influenza [63]. When the COPD virome was examined *via* nasopharyngeal swabs during COPD exacerbations, this revealed reduced abundance of bacteriophages in COPD patients suggesting skewing of the virome during infections and a possible mechanism for bacterial overgrowth [64].

Antibiotics are frequently prescribed both acute and chronically for the management of COPD and exacerbations. There is a concern that individuals with COPD may become a reservoir for ARGs. When the sputum of COPD patients was compared with healthy individuals, ARGs were 37% more prevalent in COPD sputum than in healthy controls. This excess was related to the excess bacterial burden in sputum [65]. Patients with COPD also harbour the highest diversity of resistance genes [12]. Microbial taxa associated with ARGs include *Actinomyces*, *Streptococcus* and *Prevotella*. Interestingly, resistance genes identified are associated with those found in the gut microbiota, suggesting seeding of the airway resistome may occur through aspiration of gut microbes [12].

We now have a consistent body of evidence showing the role of the microbiome in COPD, leading to considerations on how the microbiome could be targeted. For example, *H. influenzae* may be amenable to treatment with antibiotics; however, the reduction in one microorganism can lead to an increase or dominance of another [51, 59]. In bronchiectasis, another chronic lung disease, a reduction in *Haemophilus* leads to a concurrent increase in *Pseudomonas* [66]. Future interventional studies are key.

### **Bronchiectasis**

Non-cystic fibrosis bronchiectasis is characterised by irreversible dilation of bronchi, leading to chronic mucus production and poor mucus clearance. This results in a vicious cycle of persistent bacterial colonisation, inflammation, airway obstruction and progressive lung destruction. Traditional culture-based

techniques have reported colonisation with *H. influenzae*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* [67–69]. However, in up to 20% of patients, no pathogens are isolated from purulent sputum [70, 71]. NGS provides some insight into the microbiology of these patients.

There is a complex respiratory microbiome in bronchiectasis [72] and the structure within the community is extremely variable between patients [73]. Generally speaking, there is limited bacterial diversity, with no detected relationship between total bacterial load and disease state [74]. Importantly, the majority of microbiome studies in bronchiectasis have been performed on sputum samples.

Some studies have used the respiratory microbiome to phenotype and better understand disease processes. In a study which used the respiratory microbiome of patients with bronchiectasis to aide in phenotyping, the authors identified three main groups: *Pseudomonas*-dominant, *Haemophilus*-dominant and other taxa-dominant. The other taxa group mostly consisted of *Veillonella* and *Prevotella* and the majority of this group were negative on conventional cultures. Those in the *Pseudomonas*-dominant group had the lowest lung function, the most exacerbations and greatest antibiotic use. The *Haemophilus* group was associated with fewer exacerbations, but *Haemophilus* bacterial load was associated with inflammatory activation both in sputum and systemically. This suggests the host inflammatory response to *Haemophilus* may contain the infection and prevent exacerbations [74]. Notably, *Pseudomonas* was detected in 22 out of the 34 patients in the *Haemophilus* predominant group; however, they displayed no significant differences to the members without *Pseudomonas*, suggesting relative abundance is of more significance than detection. This is supported by the finding that simple PCR detection of pathogens does not influence disease severity [74].

Nontuberculous mycobacteria (NTM) are ubiquitous environmental organisms which can cause respiratory disease in patients with pre-existing lung damage, predominantly bronchiectasis. The incidence of NTM is increasing, although the cause is unclear [75]. Despite this, the role of NTM in the respiratory microbiome is not well elucidated. Mycobacteria are not accurately identified by 16S rRNA gene sequencing and although this improves with a *Mycobacterium*-biased sequencing, a poor correlation between culture positive NTM and sequencing results remains. Additionally, BAL samples from NTM-positive patients have a distinct inflammatory pattern. When microbial signatures were assessed, in NTM-positive individuals oral commensals such as *Prevotella*, *Veillonella* and *Leptotrichia* tended to co-occur, with a distinct inflammatory signature. Notably, *Mycobacterium* was in a separate cluster with no correlation to inflammatory biomarkers. This suggests micro-aspiration or failure to clear aspirated microbes may contribute to the inflammatory endotype in NTM disease [28].

The fungal mycobiome also influences clinical state in bronchiectasis. The mycobiome assessed *via* sputum in bronchiectasis is unique and dominated by *Aspergillus*, *Cryptococcus* and *Clavispora* [76]. A high proportion of patients have detectable *Aspergillus fumigatus* and/or *Aspergillus terreus*, which is not detected in healthy controls. Interestingly, geographical alterations have been demonstrated, with *A. fumigatus* detected in Singapore and *A. terreus* in Scotland. Notably, a significant number of individuals had *Aspergillus*-associated disease, ranging from colonisation to sensitisation to allergic bronchopulmonary aspergillosis (ABPA), and each had its own distinct mycobiome profile, which may be a target for future phenotyping. Furthermore, *Aspergillus*-associated disease was associated with more severe bronchiectasis, worse lung function and increased exacerbations, even in apparently clinically stable patients [76].

The virome in bronchiectasis displays a significantly higher viral load in sputum than in healthy controls and human parainfluenza virus 3 was the most prevalent. Notably, again geographic differences were observed, with higher occurrence in Asian patients compared with European patients. Analysis of bacteriophage profiles revealed a predominance of *Siphoviridae*, *Caudovirales*, *Myoviridae* and *Phycodnaviridae* families. Furthermore, when patients were clustered by exacerbation risk, patients with frequent exacerbations had a distinct bacteriophage profile [77].

Individuals with bronchiectasis frequently develop acute exacerbations, defined as an increase in symptoms for at least 48 h [78]. The mainstay of treatment is antimicrobials, with the current understanding that exacerbations are due to bacterial overgrowth [79]. The microbiome in exacerbations of bronchiectasis is highly individualised and the role of the microbiome remains unclear. Exacerbations are complex events; the microbiome is stable during exacerbations, with little change in bacterial load, diversity or abundant taxa, suggesting changes in the microbiome or bacterial overgrowth does not account for exacerbations [72, 73]. Another study, applying a multi-omic approach clustered patients into two groups. Each cluster contained a range of discriminant bacterial, fungal and viral taxa. Cluster 1 had greater microbial diversity and improved clinical outcomes, cluster 2 were more likely to be frequent exacerbators [77].



The mainstay of treatment for bronchiectasis is airway clearance and antibiotics. Chronic bacterial infections are common in bronchiectasis and are generally treated with recurrent broad-spectrum antibiotics. This in turn facilitates the development of multidrug-resistant pathogens. Similarly, long-term macrolides induce increased rates of macrolide resistance [80].

Even with all this information on the respiratory microbiome in bronchiectasis, interventional targets remain elusive. Further integrated, multi-omic and interventional studies will hopefully provide answers and improve patient outcomes.

### *Cystic fibrosis*

CF is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CF is a chronic condition associated with chronic colonisation by respiratory pathogens and understanding these microorganisms and their impact on patients is crucial.

The respiratory microbiome in CF develops over time. Until 2 years of age, the microbiome is rich in oropharyngeal taxa [81]. Eventually, the respiratory microbiome development falls into two main groups: polymicrobial (characterised by oral commensals, similar to that seen in health) or dominated by CF pathogens. Common pathogens include *Pseudomonas*, *Staphylococcus*, *Stenotrophomonas* or *Burkholderia*. Patients in the pathogen dominated group have decreased microbial diversity, worse lung function, higher degrees of inflammation and greater antibiotic use [82–85]. Assessment of the respiratory microbiome in CF has predominantly been carried out on sputum samples.

CFTR modulators have led to a major shift in CF management. Given their mechanism of action it is not surprising that they effect the respiratory microbiome. Studies examining the effect of CFTR modulators, predominantly ivacaftor, on the microbial community have been diverse, but do suggest a positive effect. A reduction in the relative abundance of *Pseudomonas* [86] with an increase in the relative abundance of oral commensals [87], leading to an increase in microbial diversity and richness with an overall reduction in total bacterial load [88] have all been demonstrated. This has been replicated with the newer, combination CFTR modulator, elexacaftor/tezacaftor/ivacaftor [89]. The exact pathogenesis is unclear, improved mucus clearance disrupting the viscous cycle of bronchiectasis, a direct anti-inflammatory or antibiotic effect of the modulators, or alterations to the environment have all been postulated [90].

The disease course of CF is regularly punctuated with acute exacerbations. Exacerbations are defined as a requirement for antibiotics due to a change in symptoms [91]. During an exacerbation of CF, the relative abundance of anaerobic genera (*Prevotella*, *Veillonella*, *Gemella*) increases with a subsequent increase in microbial diversity [92, 93]. During treatment, alpha diversity of the microbiome decreases, as does the relative abundance of anaerobes, and recovers back to baseline on recovery from the exacerbation [93]. Hence, there appears to be a stable microbiome dominated by CF pathogens and an exacerbation microbiome, dominated by anaerobic bacteria.

As with other chronic lung diseases, the virome plays an important role in the clinical course of CF. Rhinovirus is associated with worsening lung function, more exacerbations and increased bacterial colonisation [94]. Influenza and respiratory syncytial virus have also been shown to be associated with an increased risk of exacerbation [95]. The role of the mycobiome in CF is also well studied using culture-based methods, with *Aspergillus* colonisation, sensitisation and development of ABPA negatively correlated to outcomes [96]. When assessed using RNA sequencing, CF patients have a lower fungal diversity and a higher fungal burden compared with non-CF bronchiectasis, and the most prominent species was *Candida parapsilosis*.

During the lifetime of a CF patient, aggressive antibiotic therapies will be used to combat bacterial colonisation and acute exacerbations. This leads to antimicrobial resistance over time, with up to 45% of CF patients reported as colonised with a multidrug-resistant organisms [97]. The resistome of CF patients with severe disease is enhanced with ARGs compared to controls [97]. Interestingly, bacteriophage (phage) therapy, the use of lytic phages to kill infectious bacteria, has been used to treat CF patients with resistant *P. aeruginosa*, *Staphylococcus aureus*, *Burkholderia dolosa* and *Mycobacterium abscessus* with a subsequent reduction in sputum bacterial density and improved clinical status [98].

The development and now widespread use of CFTR modulators has transformed care and patient outcomes in CF. There is now a clear body of evidence that microbial dysbiosis plays a role in the pathophysiology of CF. Further studies to understand the functional potential, role of newer CFTR modulators and interventional targets are required to ultimately improve patient care.

### *Interstitial lung disease*

The interstitial lung diseases (ILDs) are a varying group of conditions, manifesting with impaired lung function and various patterns of lung fibrosis and inflammation. Some are associated with underlying conditions (such as connective tissue disease) while others are idiopathic. The most studied of these conditions is idiopathic pulmonary fibrosis (IPF). While the pathogenesis of IPF remains unclear, the underlying mechanism is thought to be a result of episodes of alveolar injury in those with defective alveolar wound-healing mechanisms [99]. One hypothesis regarding the pathogenesis of IPF has revolved around microbial insult or dysbiosis.

With this, several studies have used NGS to explore the respiratory microbiome in IPF, which has been shown to have a significantly higher bacterial burden in BAL compared with COPD or healthy controls [100, 101], where bacterial burden is negatively correlated with disease progression and survival [100, 101]. Interestingly, this association is independent of radiological features and disease extent [101]. Furthermore, the microbiome has less diverse bacterial communities and is more likely to harbour potential pathogens such as *Haemophilus*, *Neisseria* and *Streptococcus* [100]. Moreover, an increased relative abundance of *Streptococcus* is significantly associated with a reduction in progression-free survival [100]. Understanding the direct effect these microorganisms have on individuals is particularly important in understanding disease progression in IPF, however, this can be challenging. Using network analysis with bulk RNA sequencing (host transcriptome) and microbial data, genes involved in host defence response in conjunction with bacterial burden have been shown to be strongly associated with IPF, suggesting bacterial communities of the lower airways may act as a persistent stimuli and consequently lead to worse outcomes [102].

Acute exacerbations of IPF (AE-IPF) occur commonly in the disease process, with an annual incidence of 4–20% annually [103]. They are defined as an acute, clinically significant respiratory deterioration of unidentifiable cause [104]. The exact pathogenesis of AE-IPF remains unclear, however, it may represent an exaggerated lung injury in response to infection. Acute exacerbations are associated with an increase in bacterial burden in BAL samples [105]. There is a higher relative abundance of *Campylobacter* and *Stenotrophomonas* [105]. Of interest, *Campylobacter* is generally found in the gastrointestinal tract and its presence in the lower airways may suggest silent micro-aspiration of gastric contents. This strengthens the argument associating reflux and aspiration with AE-IPF.

The role of the respiratory virome in IPF is less well understood. RNA sequencing has been applied to lung tissue samples with no significant difference in the abundance of viral RNA between IPF samples and controls [106]. Viral nucleic acid has been detected in the BAL of 44% of patients with an exacerbation, compared to none with stable disease [107]. The most common viruses identified during exacerbation, *via* nasopharyngeal swab, are influenza A and human herpesvirus 6 [100]. To date, the respiratory mycobiome in IPF has not been studied.

There is a clear association between bacterial burden and prognosis in IPF, however, studies are lacking on the interaction between the respiratory microbiome and immunological tone, particularly in those ILDs associated with immune system activation.

### *Lung cancer*

Although lung cancers are a heterogenous group of malignancies, overall prognosis is poor as many present with advanced disease. Thus, lung cancer is the leading cause of cancer-related deaths worldwide. Given its wide prevalence, high mortality rate, and often delayed diagnosis, there is a need to identify biomarkers and mechanisms of progression to help diagnose, prognosticate and improve treatment options for these patients. The link between the microbiome and malignancy is an area of significant interest and prior studies have shown that distinct microbial signatures in both blood and tissues can help differentiate between cancer types and stages [108]. Thus, the relationship of the respiratory microbiome in lung cancer is of particular interest.

Several studies have shown that the lower airway microbiota differs significantly among those with lung cancer compared with healthy controls. This has been demonstrated in both human subjects as well as mouse models [109]. For example, in a mouse model of lung adenocarcinoma, where cancer was induced through deletion of p53 in lung epithelial cells, there was a significant increase in bacterial abundance and reduced microbial diversity in those with lung cancer compared with controls [110]. In human studies, we have seen that taxa commonly considered as oral commensals are differentially enriched in the lungs of patients with lung cancer. *Veillonella*, *Streptococcus* and *Prevotella* being the most abundant [111]. There are also compositional differences between different stages of lung

cancer [109, 112]. For instance, the oral commensals *Haemophilus*, *Fusobacterium*, *Gemella*, *Prevotella* and *Granulicatella* are enriched in stage IIIB–IV nonsmall cell lung cancer (NSCLC) compared with stage I–IIIA NSCLC [112].

We now understand that the host immune system has an important role in cancer development and progression. Importantly, through stimulation of the immune system microorganisms can also lead to tumorigenesis. Where individuals with enrichment of oral microbes in their lower airways have a distinct host immune phenotype with upregulation of the Th17 pathway and increased PD-1<sup>+</sup> and gamma delta ( $\gamma\delta$ ) T-cells [110–112], all which produce pro-inflammatory cytokines [13]. Moreover, specific pathways known to be important for tumour development and progression, such as the phosphoinositide 3-kinase (PI3K) and ERK pathways, have been shown to be upregulated in mouse models with lower airway dysbiosis [112]. With this relationship efforts are being made in treatment development. For instance, it has been shown that by giving antibiotics to specific pathogen-free mice with lung adenocarcinoma, tumour growth and progression can be suppressed [110]. In another study tumour burden was decreased when mice (with increased IL-17 inflammatory tone from dysbiosis) were given anti-IL-17 antibodies [112]. There is also the potential to augment treatment through the microbiome. For instance, specific taxa within the gut microbiota of patients with advanced cancers on anti-PD-1 immunotherapy were found to be overrepresented in responders when compared with non-responders [113, 114].

Thus, the lung microbiome provides a unique opportunity to identify new biomarkers in lung cancer, to better understand the role of inflammation and dysbiosis in the development and progression of malignancy, and has the potential to be used to augment and develop treatments.

#### **Future directions in the respiratory microbiome**

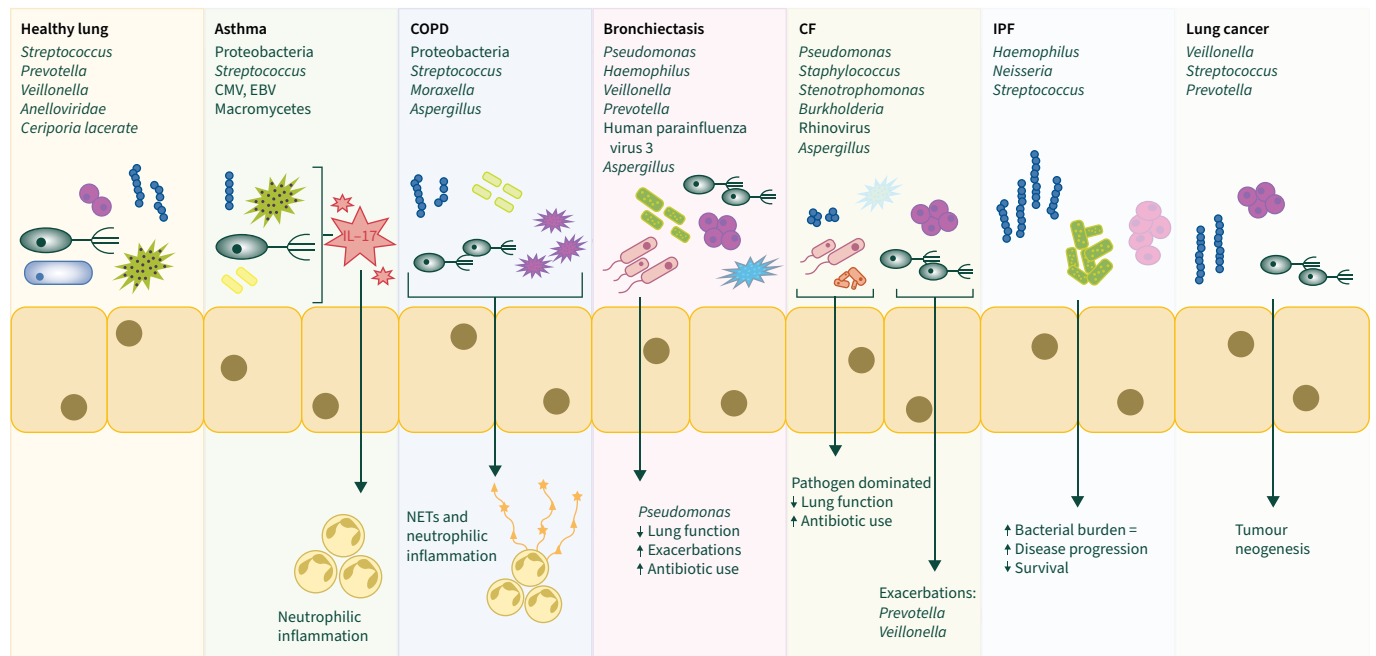
Clinical applications of the microbiome are emerging, although these are limited for the respiratory microbiome. Clinical microbiologists have utilised 16S rRNA gene sequencing as an adjunct for pathogen identification from traditional culture negative isolates. This has been successfully employed to diagnose infective endocarditis, bone and joint infections and meningitis among others. It is particularly useful for slow growing, fastidious or unusual pathogens. Interestingly, this approach has also allowed the identification of novel species [115]. Furthermore, metagenomics in real time has been applied to identify viruses, fungi and manage outbreaks, most prominently identification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the emergence of variants of concern [116]. As of yet, real time RNA transcriptomics have not been utilised.

Manipulation of the microbiome is an additional emerging clinical tool, at present limited to the gastrointestinal tract. The role of faecal microbiota transplantation (FMT) has been shown to be successful in the management of recurrent *Clostridioides difficile* infection, with emerging evidence of successfully inducing remission in ulcerative colitis [117]. Furthermore, altering the microbiome at birth with probiotics reduces the incidence of necrotising enterocolitis in low birthweight preterm infants. Interestingly, maternal FMT has been demonstrated to restore a normal gastrointestinal microbiome in infants delivered by Caesarean-section, although clinical benefit remains to be demonstrated [117]. Altering the respiratory microbiome at birth or in childhood has not yielded any clinical benefit, but is a focus of future research.

As highlighted in this review, the majority of the work evaluating the respiratory microbiome has focused on sputum samples, a poor surrogate of the lower airways. More research will be required using bronchoscopy samples and/or lung tissue samples to fully evaluate the lower airways microbiome in disease. With newer NGS techniques we can now not only identify the presence of microorganisms, but also evaluate their function and their direct effect on the host. More studies using whole genome metagenomics and in particular RNA metatranscriptomic data are required in respiratory diseases. Finally, to better understand disease progression and the role of the respiratory microbiome more longitudinal studies are key.

#### **Conclusion**

The development of culture-independent techniques has led to considerable insights into the respiratory microbiome. A concrete body of evidence describing the respiratory microbiome now exists for most disease states (figure 1), where pheno-endotyping with microbiome data can lead to the identification of high-risk patients. The emergence of multi-omic analysis has revealed interactions between the host and microbiome in some conditions, such as bronchiectasis. These interactions are a potential target for future microbiome research, with the aim of identification of treatable traits in the area of host–microbiome interaction. Further expansion of multi-omic analysis into the development of interventional studies should be a key focus of research going forward with the aim of identifying new treatment targets.



**FIGURE 1** The respiratory microbiome. CMV: cytomegalovirus; EBV: Epstein–Barr virus.

### Key points

- The respiratory microbiome encompasses bacterial, fungal and viral communities. In health, it is a dynamic structure existing across a microbial gradient from highest density in the upper respiratory tract and lowest in the lower respiratory tract.
- In health, the respiratory microbiome is characterised by Bacteroidetes, Proteobacteria and Firmicutes. *Anelloviridae* form the majority of the lower airway virome and the mycobiome consists of *Ceriporia lacerate*, *Saccharomyces cerevisiae* and *Penicillium brevicompactum*.
- The respiratory microbiome is dysbiotic in all chronic respiratory diseases studied, including asthma, COPD, ILD, bronchiectasis, CF and lung cancer.
- Dysbiosis can be related to disease severity and may be used to predict patients at risk of clinical deterioration.
- Further work is required to identify the functional potential of the microbiome and potential treatable traits.

### Self-assessment questions

- 1) What is the respiratory microbiome?
- 2) What do the terms Alpha and Beta diversity refer to?
- 3) How is the microbiome investigated?
- 4) How is the respiratory microbiome affected in disease states?

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#### Suggested answers

- 1) The microbiome is defined as the combined genomes (omic) of all the microorganisms inhabiting a specific environment such as the lung. That is to include the microorganisms and their genes.
- 2) Alpha diversity describes the mean diversity of a single sample. This can refer to the richness (number of species) or evenness (distribution of species). Beta diversity is a measure of the similarity or dissimilarity of two communities at distinct sites.
- 3) The microbiome and its functional characteristic are investigated with a combination of 16S rRNA gene sequencing, fungal internal transcribed spacer (ITS) sequencing, whole genome sequencing, RNA metatranscriptomics and metabolomics.
- 4) The respiratory microbiome is dysbiotic in respiratory disease, with each pathology exhibiting expansions in unique pathological taxa.