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Insights into human respiratory microbiome under dysbiosis and its analysis tool

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The human respiratory tract microbiome is a multi-kingdom microbial ecology that inhabits several habitats along the respiratory tract. The respiratory tract microbiome promotes host health by strengthening the immune system and avoiding pathogen infection. The lung microbiome mostly originates in the upper respiratory tract. The balance between microbial immigration and removal determines the nature of the lung microbiome. Identification and characterization of microbial communities from airways have been made much easier by recent developments in amplicon and shotgun metagenomic sequencing and data analysis techniques. In pulmonary medicine, there is a growing interest in the respiratory microbiome, which has been linked to human health and illness. However, the primary causes of bacterial co-occurrence seem to be interactions with fungi and bacteria as well as host and environmental factors. This study focused on identifying techniques and the current understanding of the relationship between the microbiota and various lung diseases.

KEYWORDS

microbiota, respiratory tract, next generation sequencing, omics tools, dysbiosis

1 Introduction

The respiratory microbiome is a heterogeneous system of microbes that includes bacteria (bacteriome), fungi (mycobiome), viruses, and phages (Schenck et al., 2016; Man et al., 2017). The host's immune response tolerates members of the microbiome in a symbiotic relationship with the host (human). In a healthy individual, bacteria represent the highly diverse and dominating member of the microbiome, but the microbiome composition may change when the immune system is compromised (Belkaid and Hand, 2014; Tomkovich and Jobin, 2016; Budden and Romani, 2025). Microorganisms create unique microenvironments, leading to variations in the microbiome at finer taxonomic levels in different regions. The makeup of these microbial communities is shaped by environmental conditions and the interactions between microbes and the host's immune system (Huffnagle et al., 2017). The normal microbiota plays a protective role by preventing the establishment of harmful pathogens. It competes with pathogens for attachment sites and nutrients, making it challenging for them to establish, multiply, and cause disease.

However, some microorganisms can cause infection when given a condition of weakened immune response or when antibiotics are used (Kumpitsch et al., 2019).

With the advent of next-generation sequencing (NGS) technology, it has been revealed that in bacteriome of the healthy human respiratory system, *Firmicutes* and *Bacteroidetes* represent the most common phyla found in the respiratory system, with *Prevotella*, *Streptococcus*, and *Veillonella* being the most common genera (Whiteside et al., 2021). Most fungi detected in the human respiratory system belong to the Basidiomycota and Ascomycota. The most prevalent genera in lung tissue include *Cladosporium*, *Eurotium*, and *Aspergillus* (Charlson et al., 2012). However, culture-independent characterization using 16S/18S rRNA gene sequence has thoroughly revealed the upper respiratory tract (URT) microbiome; the lower respiratory tract (LRT) microbiome has not been studied much (Segal and Blaser, 2014). The role of gut commensals in health and disease has been extensively researched. However, there is relatively little information available about the commensals residing in the respiratory tract, which contains a diverse microbial community that is constantly exposed to external environmental influences including COVID-19 (Emadi et al., 2024a). *Aspergillus*, *Penicillium*, and *Candida* genera are the main pulmonary fungal pathogens in patients with respiratory diseases, but several ecological species, such as *Cladosporium cladosporioides* and *Eremothecium sinicaudum*, are also present in the lung mycobiome of healthy persons (Pendleton et al., 2017). The genus *Malassezia* is overrepresented in cystic fibrosis (CF) and asthma patients (Nguyen et al., 2015). In individuals with severe Chronic obstructive pulmonary disease (COPD), *Aspergillus* species are shown to be highly prevalent (Saiman and Siegel, 2004; Huerta et al., 2014), while *Candida* spp., has been found in idiopathic pulmonary fibrosis (IPF) patients (Cuerpo et al., 2019).

2 Upper respiratory tract microbiome

The upper respiratory system comprises a network of cavities consisting of the nostril, oropharynx, and rhino-pharynx, communicating with the larynx via the Eustachian tube (Morris, 1988). The unique properties of every anatomical location in this system, including temperature, humidity, oxygen availability, and the kind of epithelial cells lining these structures, produce distinct microenvironments that eventually influence the variety of the microbiome. As a result, the microbiome varies at different taxonomic levels in each region. The interaction between the host immune system and the microbes also influences the microbiome's composition (Lambring et al., 2019; Zheng et al., 2020). Generally, the normal resident microbiota restricts the growth of harmful pathogens by limiting access to nutrients as well as indirectly by strengthening host innate and adaptive immune responses; hence, pathogens face competition for attachment sites and nutrients, making it harder for them to establish themselves, multiply, and cause disease. This competition among microbes is also a key factor in shaping the overall microbiome (McKenney and Kendall, 2016).

The colonization of the upper respiratory tract by microbiome starts at birth, and the relationship between the initial microbiome and a person's health is shaped over their lifetime through three main factors: (a) the method of delivery (whether by cesarean section or natural birth); (b) environmental influences (such as living conditions and diet); and (c) the use of antibiotics (Cicinelli et al., 2012; Gallacher and Kotecha, 2016). In the first few hours after birth, healthy full-term newborns harbor a substantial number of microbes in their respiratory tract, most of which are thought to come from the mother. During the initial week of life, the upper respiratory tract has been found to be colonized mainly by microbial genera such as *Corynebacterium*, *Staphylococcus*, and *Dolosigranulum* (Man et al., 2017; Kumpitsch et al., 2019). As time progresses, there is a subsequent rise in the presence of *Moraxella* and *Streptococcus*, and the early presence of *Corynebacterium*, *Dolosigranulum*, and *Moraxella* is strongly associated with good respiratory health (Visser et al., 2014; Bosch et al., 2016). *Streptococci* is the predominant microbiota in the healthy upper respiratory tract, with *Neisseria*, *Prevotella*, *Rothia*, and *Haemophilus* following closely behind (Cui et al., 2013; Kumpitsch et al., 2019). Bacteria that belong to the genera *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria* are the common colonizers of the mucosal surfaces of these structures (Sahin-Yilmaz and Naclerio, 2011). Studies have reported *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, *Moraxella*, and *Dolosigranulum* as the common colonizer of the nasal cavity and nasopharynx while *Streptococcus*, *Prevotella*, *Rothia*, *Veillonella*, as the common colonizer of the oropharynx. *Candida*, *Aspergillus*, *Saccharomyces*, and *Penicillium* are the common fungal genera that colonize the URT (Eidi et al., 2016; Campbell et al., 2023).

3 Lower respiratory tract microbiome

A healthy human's lower respiratory tract microbiome comprises bacteria, fungi, viruses, and bacteriophages (Li et al., 2024; Lipinski et al., 2024). The common fungal genera occurring in the lower respiratory tract of an adult human include *Aspergillus*, *Penicillium*, *Eurotium*, *Cladosporium*, *Malassezia*, *Candida*, *Saccharomyces*, and *Neosartorya* (Pulvirenti et al., 2019). Bacterial genera of LRT include *Prevotella*, *Fusobacterium*, *Streptococcus*, *Pseudomonas*, *Acinetobacter*, *Megasphaera*, *Veillonella*, *Staphylococcus*, *Sphingomonas* (Dickson et al., 2017). The microbiome in the lungs is not uniform across different parts, such as the bronchi, bronchioles, and alveoli (West, 1978; Dickson et al., 2015). Instead, it is shaped by several factors, including (a) how microbes enter the lungs (such as through inhalation of microorganisms, micro-aspiration, or the spread of mucus), (b) the mechanisms for microbial elimination (like coughing, the body's innate and adaptive immune responses and mucociliary clearance), and (c) the local environment (including nutrient availability, oxygen levels, temperature, microbial competition, and the presence and activity of inflammatory cells) (Belizario

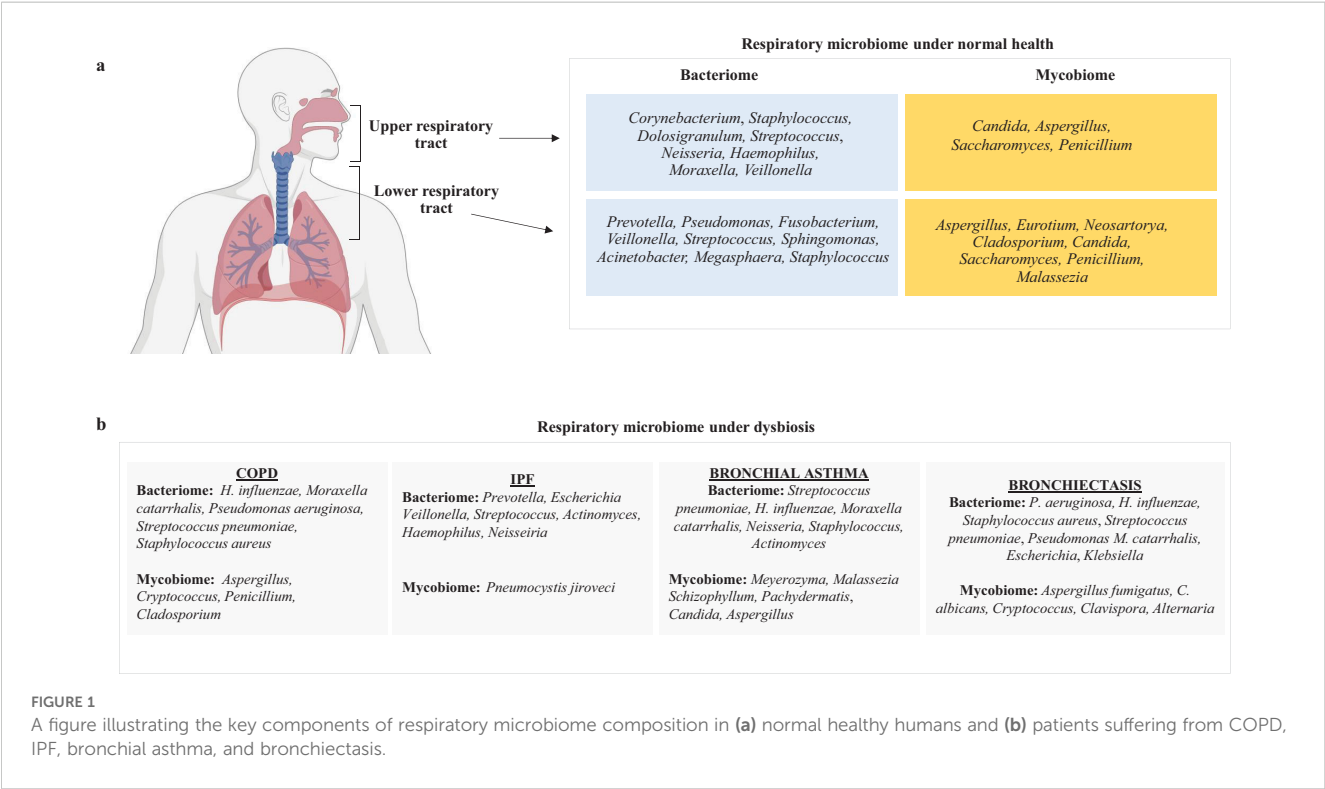
et al., 2023; Natalini et al., 2023). A decline in the lung’s ability to eliminate microbes can enhance conditions for microbial growth, leading to an imbalance in the microbiome, known as dysbiosis, which increases the risk of lung disease (Belizário et al., 2023). It should be noted that the bacteriome and mycobiome always interact with each other within the organ or the tissue in which they are localized (Rozaliyani et al., 2023) (Figure 1a).

4 Respiratory microbiome and lung diseases

Bacteria constitute most of the respiratory microbiome, while fungi are less prevalent (Zhao et al., 2023). Although mycobiome constitutes a smaller fraction of the total respiratory microbiome, there is significant diversity in the fungal species present in the respiratory tract of individuals (Weaver et al., 2019; de Dios Caballero et al., 2022; Katsoulis et al., 2024). Early research on the sputum microbiome has shown that *Ascomycota* is the dominant fungal group in COPD patients and healthy individuals (Liu et al., 2021). Many lung disorders, including cystic fibrosis, IPF, and COPD, are characterized by microbial dysbiosis, and the advancement of these diseases has been associated with a decrease in the variety of bacterial composition (Dickson et al., 2013; Garcia-Núñez et al., 2014; Puiu et al., 2024a; Angebault et al., 2025). In this section, we have tried to summarize the respiratory microbiome composition of patients suffering from lung diseases such as COPD, IPF, bronchial asthma, and bronchiectasis (Figure 1b).

4.1 COPD

Early research on the microbiome of patients with stable COPD showed a strong correlation between the presence of gram-negative enteric bacteria, neutrophilic inflammation, and elevated expression of cytokines like IL-8 and TNF in the lower airways and potentially pathogenic microbes like *Haemophilus influenza*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus aureus* (Soler et al., 1999; Bresser et al., 2000; Sethi et al., 2006; Parameswaran et al., 2009). Bacteria play an important role in the exacerbation of COPD, and it is reported that in nearly 50% of exacerbations, bacteria have been isolated from the lower respiratory tract (Sethi, 2000; Beasley et al., 2012). COPD bacteriome has been reported to vary both spatially and temporally. Changes have been observed in the transitory microbiota during exacerbations as stable COPD and exacerbated COPD microbiota have been reported to differ from each other (Tangedal et al., 2019). Enhancement with *Streptococcus*, *Prevotella*, *Staphylococcus*, and *Gemella* (strains that are part of the normal oral microbiota) was linked to abnormalities in lung function, including a worse response to bronchodilator therapy, in one of a recent study on lower airway microbiome in patients with early COPD (Opron et al., 2021). As COPD worsens, persistent inflammation weakens the innate immune system in the lungs, opening the door for more germs from old and new infections. Numerous studies have demonstrated that there is the enrichment of Gammaproteobacteria including *Moraxella* and *Haemophilus* genera, in moderate to advanced stages of COPD (Pragman et al., 2012; Sze et al., 2012) and also with the COPD



disease progression, *P. aeruginosa* has been found to become more prevalent (Beasley et al., 2012).

Respiratory mycobiome also has important links with the respiratory diseases. COPD mycobiome shows diversity and geographic variation. In a study, the effect of fungi in the development of COPD was studied using a sample of patients from three countries (Malaysia, Singapore, and Hong Kong) (Tiew et al., 2020). COPD mycobiome was diverse in stable COPD, and different genera, namely *Alternaria*, *Cryptococcus*, *Mycosphaerella*, *Trametes*, *Aspergillus*, *Penicillium*, *Wickerhamomyces*, and *Cladosporium*, were found only in diseased lungs and not in healthy lungs (Martinsen et al., 2021). Also, microbiome enhancement with *Penicillium*, *Curvularia*, and *Aspergillus* species may be associated with a greater mortality rate and higher exacerbation rate, as revealed by clustering analysis. Moreover, a reduction in fungal diversity has been linked to higher mortality rates in COPD patients. Elevated levels of the fungal genera *Penicillium*, *Trametes*, *Lodderomyces*, and *Cladosporium* are associated with poor survival rates (Tiew et al., 2021).

4.2 Idiopathic pulmonary fibrosis (IPF)

IPF is a persistent, lethal lung parenchymal remodeling disease with an unclear cause (Barratt et al., 2018; Zhang and Wang, 2023). *Haemophilus*, *Streptococcus*, *Neisseria*, *Prevotella*, *Escherichia*, and *Veillonella* are the common bacterial genera that have been identified in the lungs of individuals suffering from IPF (Molyneaux et al., 2014; Fastrès et al., 2017). The population of *Streptococcus* species also increased in IPF patients. Employing culture-independent methods, an elevated bacterial burden in bronchoalveolar lavage fluid, which enables the identification of microbiota specific to the lower lungs of IPF patients, was observed compared to controls (Schneeberger et al., 2019). It is reported that *Firmicutes* dominate in the lower respiratory tract of IPF patients, while *Proteobacteria* is less prevalent. At the genus level, *Streptococcus*, *Actinomyces*, *Haemophilus*, *Neisseria*, and *Veillonella* were reported to be abundant in the lungs of IPF patients (Invernizzi et al., 2021). A correlation between mortality rates in IPF patients and the amount of bacteria in their lungs was established (Molyneaux et al., 2014). Increased levels of granulocyte colony-stimulating factor, IL-1 β , IL-1Ra, epidermal growth factors, CXCL8, and macrophage inflammatory protein-1 α , as well as aberrant alveolar inflammatory and fibrotic cytokines, are linked to lung microbiota diversity in IPF (O'Dwyer et al., 2019). Furthermore, a positive correlation was found between alveolar concentrations of IL-6 and the relative abundance of the lung *Firmicutes* phylum and a negative relationship between alveolar IL-12p70 and the relative abundance of the lung *Proteobacteria* phylum (O'Dwyer et al., 2019). The fungal composition of IPF lungs has not been studied much and remains a subject that needs to be explored. However, a study has reported higher colonization of *Pneumocystis jiroveci* to be associated with IPF patients (Vidal et al., 2006).

4.3 Bronchial asthma

Dysbiosis of the respiratory microbiome is an important factor in the development of asthma. A decrease in the bacteriome diversity is reported in asthma and is associated with the disease severity (Huang et al., 2015; Barcik et al., 2020; Valverde-Molina and García-Marcos, 2023). *Moraxella*, *Neisseria*, *Staphylococcus*, *Actinomyces*, and *Haemophilus* are among the prominent bacterial pathogens associated with asthma exacerbations (Hilty et al., 2010; Mthembu et al., 2021). Other bacteria that have been reported are *Gammaproteobacteria* and *Proteobacteria*, and respiratory commensal bacteria like *Prevotella* and *Veillonella* are found to be less dominant (Hilty et al., 2010). In the lower and upper respiratory tract of children with asthma disease, three bacterial species, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae*, are predominant (Thorsen et al., 2022). In patients that are hospitalized with atopic asthma, the tracheal microbiome has revealed some prevalent bacteria that feature *Fusobacterium*, *Sphingomonas*, *Haemophilus*, *Porphyromonas*, and *Neisseriaceae*, while *Bacteroides* and *Lactobacillus* were found in low levels (Durack et al., 2017). T-helper cell type 2 (Th2)-high and Th2-low are the two inflammatory endotypes associated with asthma (Wenzel et al., 1999; Kuruvilla et al., 2019). It is reported that fungal diversity is significantly lower in Th2-high individuals than in Th2-low individuals, and fungal genera associated with type 2 inflammation are *Aspergillus*, *Cladosporium*, *Alternaria*, *Mycosphaerella*, *Fusarium*, *Penicillium*, *Wallemia* and *Trichoderma* (van Woerden et al., 2013). Other fungal genera reported in asthmatic lungs are *Meyerozyma*, *Schizophyllum*, *Malassezia*, and *Candida* (Chung, 2017). The lung microbiome activates Th2 cells and other pathways and induces chronic inflammation, which may aggravate the progression of asthma. During this inflammation, the growth of some bacterial colonies may be stimulated, and microbial dysbiosis may occur.

4.4 Bronchiectasis

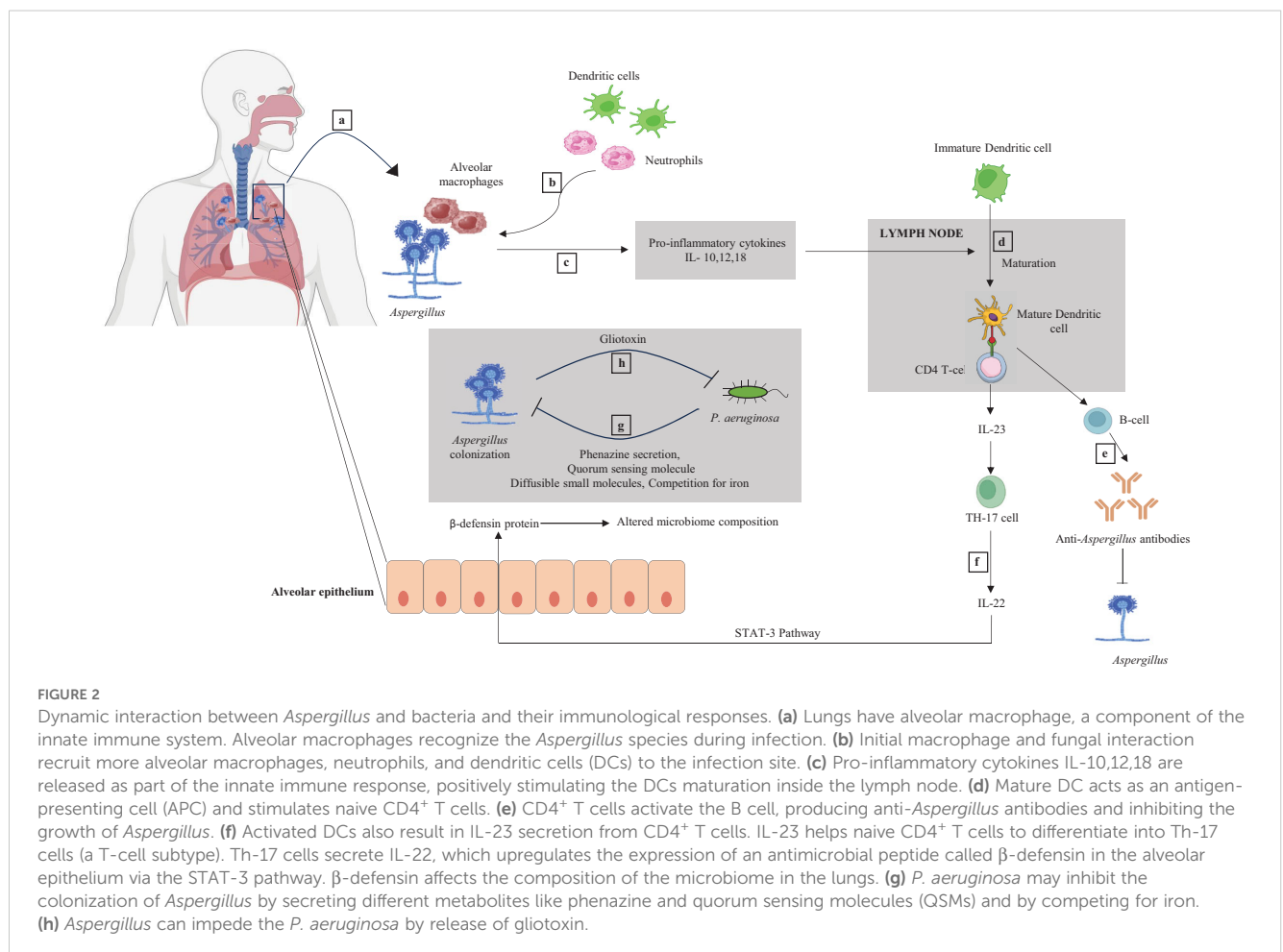
Bronchiectasis is a long-term inflammatory lung condition marked by the abnormal dilation of one or more airways (McShane and Tino, 2019; Chen et al., 2024). In these patients, reduced mucociliary function leads to mucus accumulation in certain areas of the airways, which fosters the persistence of fungi and bacteria (Máiz et al., 2015; Máiz et al., 2018). The most prevalent bacterial species identified in bronchiectasis are *P. aeruginosa* and *Haemophilus influenzae*. However, other species have also been identified, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Escherichia* spp., and *Klebsiella* spp (Chalmers et al., 2014). With a large number of studies being conducted on the respiratory microbiomes, it is now well understood that these diseases are characterized by either dysbiosis (state of the dominance of certain taxa) or loss of diversity of bacterial and fungal species in the microbiome. It is

reported that the diseased “dysbiotic” airways are primarily dominated by proteobacteria, such as *Pseudomonas* and *Haemophilus*, which are linked to increased neutrophil-mediated inflammation and exacerbations (Tunney et al., 2013). However, there is a subgroup of patients whose microbiota is dominated by genera *Firmicutes* and *Veillonella* who experience frequent exacerbations despite having lower levels of neutrophilic inflammation (Rogers et al., 2014). A reduction in microbial diversity, particularly marked by a dominance of *Pseudomonas*, was linked to an increased risk of severe bronchiectasis, more frequent episodes of worsening symptoms, and higher mortality rates (Dicker et al., 2021).

Aspergillus fumigatus and *Candida albicans* have been identified as major and common fungus species in the bronchiectasis mycobiome (Mortensen et al., 2011; Chalmers et al., 2014). *Aspergillus* is the principal species distinguishing healthy people from bronchiectasis patients, and its presence has been associated with exacerbations. Thus, *Aspergillus* may have a considerable effect on airway inflammation. A study reported that fungal genera, namely *Cryptococcus*, *Alternaria*, *Clavispora*, *Botrytis*, and *Aspergillus*, were specifically identified only in bronchiectasis patients and not in the healthy controls. However, *Saccharomyces*, *Candida*, and *Penicillium* were reported in both bronchiectasis patients and healthy controls (Aogáin et al., 2018). *Aspergillus*

spp., can strongly induce immunological responses such as interleukin-22 at the level of the respiratory mucosa. This, in turn, controls the production of defensins, which are peptides with antimicrobial action that affect the makeup of the pulmonary bacterial microbiome, which includes *P. aeruginosa*. *Aspergillus* spp., also cause an increase in lung macrophages and a Th2 and Th17 response (Kim et al., 2014; Baker and Dickson, 2025) (Figure 2).

We provided an overview of the human respiratory microbiome and the ways in which the microbiome of a healthy person varies from that of people with different respiratory conditions in the first part. It makes more sense to research the human pulmonary microbiome along with techniques used to identify the bacteria species. As a result, we have also attempted to provide an overview of the techniques that have enabled the thorough investigation of the human microbiome in the following section. Different respiratory diseases have distinct microbial profiles, with certain species being more numerous in one while being missing or less prevalent in another. These techniques have aided in the identification of microbial interactions and the classification of both pathogenic and commensal species in the human microbiome. An overview of the techniques employed in the investigation of the human microbiome, including some that have received recent attention, is given in this literature. The human



microbiome is comprehensively investigated using the “metagenomics” technique. Among these are metagenomics, which examines genes, metatranscriptomics, which analyzes transcripts, metaproteomics, which studies the proteome, metabolomics, which analyzes metabolites produced by the microbiome, and 16S rRNA gene sequencing, which determines the phylogenetic marker in bacteria and the internal transcribed spacer (ITS) region in fungi (Takeuchi et al., 2025a). Another new and effective technique is single cell genomics, which is mostly used to examine the low abundance of microorganisms in a microbiome sample and also aids in examining the heterogeneity even within a single strain. The relevant section contains a detailed discussion of the procedures.

5 Methods to study human microbiome

Currently, several methods are available to study the human microbiome, and in this review, we have tried to emphasize how these methods have been used to study different aspects of the human microbiome along with advantages and disadvantages of these methods (Tables 1, 2).

5.1 Gene marker analysis

Targeted sequencing is the approach most frequently employed to examine the microbiome. Certain genes are sequenced, including the 16S ribosomal RNA (rRNA) gene for bacteria and the internal transcribed spacer (ITS) region for fungi. Determining and recognizing a unique sequence is one of the challenges in marker gene investigations present (Galloway-Peña and Hanson, 2020; Pei et al., 2023). Hypervariable areas in both the ITS and 16S rRNA genes can alter quickly and even vary within a single cell (Yang et al., 2016). Additionally, it can be difficult to distinguish between different taxa and define a unique sequence when they share similar gene sequences, as in the case of *Escherichia* and *Shigella* (Větrovský and Baldrian, 2013). Therefore, a method of binning sequences called operational taxonomic units (OTUs) is now being used. The 16S rRNA gene is amplified and sequenced to create the most common type of comprehensive microbiome taxonomic data, and the acquired sequences are typically clustered into OTUs based on the overall percent similarity of the sequences. The relative abundance of each OTU in the sample is then determined (Ju and Zhang, 2015). Each OTU's taxonomy can be deduced by grouping the reads with reference sequences of known taxonomy or by using a classification algorithm. This algorithm estimates the probable taxonomy of each OTU or individual read (Ju and Zhang, 2015; Kopylova et al., 2016).

Although the targeted amplicon technique only provides information about bacteria within a community, it can also be adapted to explore fungal species. Amplification of the fungal ITS regions is the most popular method. The two ITS regions called ITS1 and ITS2, are situated between the 18S and 5.8S rDNA and

5.8S and 26S rDNA genes, respectively (Schoch et al., 2012). The genetic locus containing the regions ITS1 and ITS2, which encode for the nonfunctional RNA transcribed during rRNA synthesis, as well as the 18S, 5.8S, and 28S rRNA genes, have been the main targets for culture-independent analyses and database generation (De Filippis et al., 2017). The utilization of pyrosequencing in NGS techniques, along with the construction of clone libraries from the 18S, 5.8S, and 28S regions, along with ITS1 and ITS2, have unveiled a pool of undisclosed variety of fungi in the microbiome, both culturable and nonculturable (Siqueira et al., 2012; Akaçin et al., 2022). However, the two most common problems faced in ITS sequencing are: first, in contrast to bacterial 16S sequences, ITS sequences lack a well-established database. Second, fungal ITS sequences from various species can vary greatly in size and sequence content (Abarenkov et al., 2010). Also, PCR amplification can result in biased abundance comparisons if the ribosomal genes of some taxa are poorly amplified (Walker et al., 2015).

In a study by Morris et al. (2013), gene marker analysis method was applied to study and compare the respiratory microbiome of the healthy non-smokers and smokers. Bacterial species like *Methylobacterium*, *Enterobacteriaceae*, *Ralstonia* and *Haemophilus* were significantly reduced in the lung microbiome compared to the value predicted by the neutral model (Du et al., 2024). The lung bacterial population of smokers and non-smokers was found to be same, however, the mouth bacterial community differed in species such as *Porphyromonas*, *Gemella* and *Neisseria*. Thus, the gene marker analysis method helped to reveal that smoking does not significantly alter the lung microbiome (Morris et al., 2013). Charlson et al., 2012 also used gene marker analysis method to study the aberrant fungal and bacterial microbiome of lung transplant recipients. Using the fungal internal transcribed spacer and bacterial 16S rDNA sequencing, the bacterial and fungal species were profiled. URT was sampled by oropharyngeal wash and lung by Bronchoalveolar lavage (BAL). A high bacterial burden was observed in the BAL in lung transplant recipients as compared to the control group. Fungal population in BAL was dominated by *Aspergillus*, while *Candida* was found to be dominant at both the sites (OW and BAL) (Charlson et al., 2012).

5.2 Single-cell genomics

The single-cell genomics technique involves isolating a single microbial cell from a heterogenous microbiome sample and sequencing each individually isolated cell of a microbiome to obtain the information. This technique is helpful in studying low abundant microorganisms present in a microbiome sample, which are not covered well by common sequencing methods like 16S rRNA gene amplification and sequencing (Hatzenpichler et al., 2020; Bowers et al., 2022). Microbial cell populations can show heterogeneity in gene expression; thus, they can be heterogeneous even within a single strain; this type of microdiversity can be studied using a single-cell genomics approach (Elowitz et al., 2002). However, isolating single microbial cells from a heterogenous

TABLE 1 Methods utilized to study the human microbiome.

| Method of study | Microbiome studied | Purpose of the study | Results | References |
|--|--|---|--|---------------------------|
| 1. GENE MARKER ANALYSIS | | | | |
| Sequencing of 16S rRNA | Respiratory microbiome | To study the characteristics of sputum microbiota in patients having acute exacerbation of other disease states of COPD | Bacterial diversity was decreased in patients having acute exacerbated COPD and bacterial composition differed from patients with stable COPD and healthy controls | (Chang et al., 2021) |
| | Respiratory microbiome | To study the bacterial profile of respiratory microbiome in healthy smokers and non-smokers | The relative abundance of <i>Neisseria</i> , <i>Gemella</i> , and <i>Porphyromonas</i> was reduced in smokers. | (Long et al., 2020) |
| | Gut microbiome | To study the profound effect of ciprofloxacin antibiotic on gut microbiome of human | Approximately 30% of the gut's taxa experienced relative abundance changes due to Ciprofloxacin treatment. | (Jiang et al., 2022) |
| Sequencing of Internal Transcribed Spacer (ITS) region | Respiratory microbiome | To study aberrant mycobiome in the respiratory tract after lung transport | The subjects with lung transplants showed differences in the microbial load and community composition and had reduced fungal diversity when compared to healthy control groups. | (Wilmanski et al., 2019a) |
| | Gut microbiome | To characterize the gut fungal microbiome (mycobiome) of the Human Microbiome Project (HMP) cohort; ITS2 region sequencing. | Results demonstrated that gut mycobiome is less diverse than gut bacteriome and <i>Saccharomyces</i> , <i>Malassezia</i> , and <i>Candida</i> are the most prevalent yeast genera in this cohort. | (Vidal et al., 2006) |
| 2. SINGLE CELL GENOMICS | Human microbiome (skin and gut microbiota) | To reconstruct strain-specific genomes from the human microbiome using a combined approach of single-cell genomics and metagenomics. | A high-quality draft genome was produced from both mock and human microbiome samples using single-cell metagenomics method genes. | (Arikawa et al., 2021) |
| | Oral microbiome | Isolation and amplification of a rare and uncultivated bacterial cell (TM7 phylum; cells having rod-like morphotype) from oral microbiota of human and sequenced their DNA gain genetic insights. | Individual TM7 cells' genome amplification allowed sequencing and assembly of more than 1,000 genes, and the physiology of members of this phylum was studied. | (Marcy et al., 2007) |
| 3. SHOTGUN METAGENOMICS | Gastric microbiome | To assess the role and makeup of the gastric microbiota in advanced gastric adenocarcinoma (GC) as well as in superficial gastritis (SG). | The most prevalent taxa identified in GC are commensals or opportunistic pathogens also present in the oral cavity. Pathways for lipopolysaccharide (LPS) and L-arginine biosynthesis were enhanced in GC, while those for short-chain fatty acid (SCFA) fermentation and branched-chain amino acid metabolism were more enriched in SG. | (Hu et al., 2018) |
| | Gut microbiome | Profiling of human gut microbiome in COVID-19 patients to study change caused during the disease | Four bacterial species were identified to be unique to COVID-19 patients, namely <i>Prevotella bivia</i> , <i>Streptococcus thermophilus</i> , <i>Fusobacterium ulcerans</i> and <i>Bacteroides oleiciplenus</i> | (Li et al., 2021) |
| 4. METATRANSCRIPT -OMICS | Oral microbiome | Metatranscriptomic analysis of the human oral microbiome in both healthy and diseased states. | Microbiota associated with periodontal disease are more similar to each other than those linked to healthy states. Average microbial composition for both healthy and disease-related plaque was identified. The metabolic gene expression of different species showed significant variability among patients. | (Jorth et al., 2014) |
| | Lung microbiome | To study the metatranscriptome associated with human lung cancer. | Fifty taxonomic groups were identified, out of that seven taxonomic groups were linked to lung cancer patient's survival. Cytotoxic T-cells, naive T helper cells, dendritic cells, and central memory T helper cells were linked to improved overall survival, whereas pro-B-cell enhancement indicated poorer survival outcomes. | (Chang et al., 2021) |

(Continued)

TABLE 1 Continued

| Method of study | Microbiome studied | Purpose of the study | Results | References |
|-------------------|--------------------|--|--|---------------------------|
| 5. METAPROTEOMICS | Gut microbiome | To investigate the functional roles of the human gut microbiome in colorectal cancer by using metaproteomics | Healthy individuals had 17% more identified proteins than colorectal cancer (CRC) patients, indicating that CRC patients exhibit lower microbial protein diversity. Taxonomic abundance differences were observed in the gut microbiome of colorectal cancer patients compared to healthy controls, with <i>Methanobacteriaceae</i> , <i>Sporolactobacillaceae</i> , and <i>Desulfohalobacteres</i> being abundant in the CRC patients | (Long et al., 2020) |
| | Oral microbiome | To perform a detailed metaproteomics analysis of the oral microbiome to identify specific protein profiles associated with lung cancer | Compared to the conventional metaproteomics approach, interfering peptides decreased by 52.87%, while bacterial peptides and species increased by 94.97% and 44.90%, respectively. The decrease in the interfering peptide increased the reproducibility. | (Jiang et al., 2022) |
| METABOLOMICS | Gut microbiome | To study alpha diversity of the human gut microbiome using blood metabolome | Shannon diversity predictions effectively represented shifts in gut microbial diversity related to gastrointestinal health, underscoring a strong connection between the structure of the gut microbiome and host physiology. | (Wilmanski et al., 2019a) |

sample is difficult as bacteria often show aggregation (Nwoko and Okeke, 2021). Moreover, the bacterial cell wall is required to be permeabilized as it poses difficulty in sequencing single cells (Blattman et al., 2020). Single cells are isolated using techniques like fluorescence-activated cell sorting (FACS), micromanipulation, and microfluidics (Tolonen and Xavier, 2017). FACS is a high throughput technique that can isolate single cells based on their size, internal complexity, and fluorescence. Micromanipulation involves the use of micropipettes and an inverted microscope, and single cells are isolated from a heterogeneous mixture. It is a time-consuming and labor-intensive method (Blainey, 2013). Microfluidics offers high throughput isolation of single cells and individual genome barcoding (Leung et al., 2012). Unlike metagenomics, single-cell genomics does not depend on the clonality of microbial populations; instead, it retrieves genome sequences from individual cells. Amplification biases may occur during DNA amplification, which results in incomplete genome sequences. Therefore, the single-cell genomics technique is highly accurate when the single-cell sequencing data are co-assembled as it minimizes the gaps and errors that may present in the individual single-cell amplified genomes (Kogawa et al., 2018).

5.3 Shotgun metagenomics method

It is a powerful non-culture-based technique. In shotgun metagenomics, all of the microbial genomes in a sample are captured by means of untargeted sequencing techniques (Quince et al., 2017; Sharpton, 2014). Depending on the sequencing depth, it is possible to identify every DNA genome that is present in a sample, and since complete genetic repertoire is extracted, it can determine different taxa that are present in the sample with their relative abundance. It can also decipher information about the gene coding sequences (Qin et al., 2010; Xia et al., 2011). In this method, DNA is extracted from all cells in the community and broken into small fragments rather than targeted and amplified in a specific genomic region. These fragments are then sequenced separately. This produces DNA sequences, or “reads,” corresponding to various genomic regions from the many organisms present in the sample, including non-microbial species. Some of the sequences will come from coding regions that reveal the biological functions encoded in the genomes, while others come from taxonomically important regions like 16S rRNA (Sharpton, 2014). Like any other technique, shotgun metagenomics also has certain disadvantages. This technique presents complex and large data; the analysis process is difficult, and it is often difficult to determine the genome from where a particular read originated. Additionally, while studying the microbiome, metagenomes must be separated from contaminating host DNA (Chew and Holmes, 2009). The adaptability of this approach is restricted by the high expense of sequencing each sample, in addition to the technical difficulties of DNA shearing, library creation, and bioinformatics pipelines (Usyk et al., 2023). Also, finding and eliminating contaminants from metagenomic sequences is particularly challenging, and it is hard to assess which read has resulted from a contaminant metagenome.

TABLE 2 Pros and cons of the methods used to study human microbiome.

| Method of study | Pros | Cons | References |
|-------------------------|---|--|---|
| 1. GENE MARKER ANALYSIS | Cost-effective and gives insight into microbiome diversity and abundance. | Less information is revealed for low abundant members and lacks functional insight, PCR can result in a biased abundance composition. ITS sequences lack a well-established database. | (Nilsson et al., 2006; Kembel et al., 2012; Walker et al., 2015) |
| 2. SINGLE-CELL GENOMICS | Useful for the study of microdiversity within a single strain and low abundant members of the microbiome. | Aggregation of bacterial cells, bacterial cell wall challenges sequencing of single cells. | (Elowitz et al., 2002; Hatzenpichler et al., 2020; Bowers et al., 2022) |
| 3. SHOTGUN METAGENOMICS | All microbial genomes can be captured, allowing the study of different taxa, their abundance, and gene coding sequences. | Difficult to determine the genome from where a particular read has originated, high sequencing cost. | (Xia et al., 2011; Sharpton, 2014; Quince et al., 2017); |
| 4. METATRSCRIPTOMICS | Identify the transcriptionally active organism's taxonomy and the function of their expressed genes. | Requires high-quality RNA, fails to sense short-lived response generated against stimuli as mRNA has a short half-life, and requires an integrative omics approach as mRNA presence may not imply the presence of protein. | (Bashiardes et al., 2016) |
| 5. METAPROTEOMICS | Determine microbiome composition, physiology, and metabolism of each individual microorganism, decipher the host-microbiome interactions | Lack of databases, bioinformatics tools, poor yield of proteins, poor peptide identification. | (Kleiner, 2019; Wang et al., 2020; Salvato et al., 2021) |
| 6. METABOLOMICS | Profiles of metabolites produced within a microbiome help in studying host-microbiome interaction. Can detect both endogenous and exogenous substance | Origin of the metabolite is not known | (Lamichhane et al., 2018; Zierer et al., 2018) |

If a metagenomic contaminant's genome is enriched for rare genes, it can skew assessments of community genetic diversity. This is particularly true if the contaminant is very prevalent or has a big genome (Schmieder and Edwards, 2011).

5.4 Metatranscriptomics

Metatranscriptomics can be applied to study the gene expression of the bacterial communities present at a site within or on the human host. Gene expression profiling can be done for both culturable and non-culturable bacteria. The data of metatranscriptomic analysis complement data obtained in metagenomics analysis since this helps to precisely identify which genes are transcribed out of all the genes that were annotated in the metagenomics analysis. It elucidates the extent to which the genes are transcribed and, therefore, helps in determining the function of these genes (Franzosa et al., 2014). For metatranscriptomic analysis of the microbiome, total RNA is isolated from the bacterial community colonizing a particular niche. Only up to 5% of all RNA species are prokaryotic mRNA, the majority being the 16S and 23S rRNAs and tRNAs (Peano et al., 2013). Eukaryotic mRNAs contain a poly-A tail, which can be used to obtain cDNA by using oligo dT primers, while prokaryotic mRNAs lack a poly-A tail. Thus, their isolation is not simple. Prokaryotic mRNAs can be isolated using probes attached to a magnetic bead. They anneal to their complementary target sequences, and magnetic beads help in their isolation (Sultan et al., 2014). RNase inhibitors are added

during the extraction process to prevent their degradation from ribonucleases. The RNA obtained is cut into smaller fragments, and cDNA is made using oligo dT primers. Adaptors are ligated to cDNA ends, generating a representative library that is subjected to amplification and sequencing (Bikel et al., 2015). Illumina sequencing methodologies are commonly incorporated in shotgun metagenomics and metatranscriptomics as it is a high throughput technique and comparatively cheaper (Galloway-Peña and Hanson, 2020). The sequenced mRNAs are called RNA sequence reads, and to identify the transcriptionally active organism's taxonomy and the function of their expressed genes, these RNA sequence reads are mapped to different genomes and pathways (Galloway-Peña and Hanson, 2020). Metatranscriptomics data obtained from a microbiome sample can be aligned and assembled using bioinformatics tools like SOAPdenovo (Xie et al., 2014). Certain factors that limit the use of metatranscriptomics include the requirement of high-quality RNA from the microbiome sample. mRNA has a short half-life and is prone to degradation by nucleases; therefore, sometimes, it fails to sense the responses generated against stimuli. Moreover, the presence of mRNA may not always imply the presence of protein, and an integrative omics approach is required to study the microbiome sample better (Bashiardes et al., 2016). In a study by Chang et al. (2021), metatranscriptomics method was employed to analyse the human lung microbiome in patients with lung cancer. RNA-Seq was performed for the investigation of the human lung cancer metatranscriptome. The study revealed that a number of bacterial species that significantly associated with the prognosis were

enriched in the lung cancer tissues (Emadi et al., 2025a). It included *Staphylococcus* spp., *B. diminuta*, *M. franklinii*, *E. cloacae*, *M. chelonae*, *A. radioresistens*, *B. megaterium*, *R. erythropolis* and *P. aeruginosa*.

5.5 Metaproteomics

Metaproteomics deals with studying proteins expressed by a microbiome sample. It can also be used to study the proteins expressed within any microbiome community at a given time (Wilmes and Bond, 2004). However, to correctly identify the peptide and to analyze pathways, metaproteomics requires databases and libraries created using metagenomics (Lai et al., 2019). It is also helpful in determining the composition of the microbiome, physiology, and metabolism of each microorganism that is part of the microbiome. Using metaproteomics, the host gene expression and the microbial gene expression can be studied, and thus, it can help decipher the host-microbiome interactions that may be involved in causing disease (Salvato et al., 2021). Metaproteomics has not been used as much as metatranscriptomics and metabolomics because of a lack of databases, bioinformatics tools, poor yield of proteins, and poor peptide identification (Kleiner, 2019; Wang et al., 2020). It takes access to both an enriched protein sequence database and instrumentations like high-resolution liquid chromatography (HRLC) and mass spectrometry to perform metaproteomics of a sample. A protein database predicts a theoretical spectrum for a sample to which the experimentally obtained mass spectra are compared, provided that the database has the expected protein sequence. A successful metaproteomic study results in good protein extraction efficiency, separation efficiency, and unambiguous identification (Hettich et al., 2013; Tanca et al., 2016; Rechenberger et al., 2019).

5.6 Metabolomics

Metabolomics analyzes the metabolites produced by the microbiota and studies the interaction of these metabolites with both the host metabolism and the microbiota (Lamichhane et al., 2018). Bacteria produce small molecules as part of their metabolic activity; for example, *P. aeruginosa* secretes different metabolites like phenazine and quorum sensing molecules, which may inhibit the colonization of *Aspergillus*. Host antifungal immunity can be modulated by molecules released by microbiota during their metabolic activity. *P. aeruginosa* can produce metabolites in the CF lung that significantly affect how it interacts with *A. fumigatus*. Recently, a study of metabolomics using molecular networking showed that xenobiotics, specialized metabolites from *P. aeruginosa*, and host sphingolipids make up the chemical composition of CF sputa (Quinn et al., 2016). However, the microbial metabolites were not the same as those produced by laboratory cultures, and the *P. aeruginosa*-derived quinolone signal was not present in sputum, even in the presence of its precursor

molecules, but was easily identified from cultured strains. This reflects that the signals provided by the CF lung chemical environment affect the *P. aeruginosa* metabolism (Mowat et al., 2010; Quinn et al., 2016).

These metabolites can be detected using metabolomics, and techniques like mass spectrometry (MS) are often used to identify known metabolites (Zierer et al., 2018). A complex biological sample contains diverse metabolites that can be sensed and quantified using mass spectrometry because of its high sensitivity (Aksenov et al., 2017). Tools used for metabolomic studies can detect both endogenous and exogenous substances. MS can be used for the detection and quantification of metabolites released by the microbiome with the aid of different samples, including solids (involves detecting the metabolites directly from the surface) or volatiles (microbial volatile organic compounds released by the microbiome) (Shih et al., 2014; Weisskopf et al., 2021). Biotyping is a common strategy to identify microbial metabolites from a clinical sample. It utilizes the Matrix-assisted laser desorption/ionization-Time of flight (MALDI-TOF) technique to profile the ribosomal proteins present in bacteria colonies, and the data obtained is compared with the MS pattern obtained from a clinically important microorganism that has been well characterized. This helps in the taxon identification of the sample (Freiwald and Sauer, 2009). MS Imaging (MSI) has also been used to study the metabolic exchange between two or even more microorganisms (Watrous et al., 2013).

6 Conclusion

It has only just been apparent how the respiratory microbiomes contribute to colonization resistance and immune system activation despite long being overlooked. However, to completely understand the human respiratory microbiome ecosystem's relationship to human physiology and illness, it is necessary to understand its mechanism of interaction. An important part of this microbial ecology comes from the oral-lung and gut-lung axis (Bongers et al., 2024). A disturbed respiratory microbiota is a hallmark of four chronic respiratory diseases: asthma, COPD, CF, and IPF. Each of these illnesses has been found to have unique mucosal microbial signatures. Pulmonary medicine might be revolutionized by identifying small molecule metabolites originating from the microbiome and how they affect the host's inflammatory and immunological responses. Incorporating the respiratory microbiome into pathophysiologic concepts of chronic disease will further our understanding of lung respiratory microbiomes and the development of new therapeutic approaches.

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Conflict of interest

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