



The microbiome in respiratory medicine: current challenges and future perspectives

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The respiratory system bacterial community is dominated by specific phyla that change in chronic respiratory diseases <http://ow.ly/j68Z30967DB>

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ABSTRACT The healthy lung has previously been considered to be a sterile organ because standard microbiological culture techniques consistently yield negative results. However, culture-independent techniques report that large numbers of microorganisms coexist in the lung. There are many unknown aspects in the field, but available reports show that the lower respiratory tract microbiota: 1) is similar in healthy subjects to the oropharyngeal microbiota and dominated by members of the Firmicutes, Bacteroidetes and Proteobacteria phyla; 2) shows changes in smokers and well-defined differences in chronic respiratory diseases, although the temporal and spatial kinetics of these changes are only partially known; and 3) shows relatively abundant non-cultivable bacteria in chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, cystic fibrosis and bronchiectasis, with specific patterns for each disease. In all of these diseases, a loss of diversity, paralleled by an over-representation of Proteobacteria (dysbiosis), has been related to disease severity and exacerbations. However, it is unknown whether dysbiosis is a cause or a consequence of the damage to bronchoalveolar surfaces.

Finally, little is known about bacterial functionality and the interactions between viruses, fungi and bacteria. It is expected that future research in bacterial gene expressions, metagenomics longitudinal analysis and host-microbiome animal models will help to move towards targeted microbiome interventions in respiratory diseases.

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Introduction

Healthy lungs have been traditionally considered to be a sterile organ because standard microbiological culture techniques consistently yield negative results [1]. In the last decade, however, the use of culture-independent molecular techniques has demonstrated that this dogma is wrong, and that large numbers of microbiological organisms, including bacteria, fungi and viruses, collectively known as the microbiome, coexist in the lungs of healthy subjects and patients with respiratory diseases [2, 3], challenging our understanding of the microbiology in respiratory medicine [3]. Indeed, addressing the nature of the relationships between the lung microbiota and respiratory epithelial surfaces appears to be one of the most promising research fields in respiratory medicine [1]. For instance, a large body of evidence now supports the concept that abnormal regulation of host–microbiota crosstalk in different organs and at different body surfaces may play an important pathogenic role in several chronic inflammatory disorders [4–7]. As a consequence, there is growing interest in determining the potential value of the characterisation of airway microbiome composition as a prognostic marker or as an element capable of guiding therapy in several respiratory diseases [3]. This manuscript reflects the current level of knowledge on the respiratory microbiome (see Box 1 for the current terminology), and its unspecificities can be intrinsically related to the heterogeneity of the clinical stratification of respiratory diseases that is currently in use. With these considerations in mind, the Barcelona Respiratory Network organised an international, multidisciplinary workshop on June 3rd, 2016, to discuss and identify research challenges, priorities and gaps in the field, as well as to examine future directions and implications both for patients and healthcare systems. The discussions that took place there, as well as the main conclusions of the workshop, are summarised below. Full presentations were video-recorded and are freely available online at the Barcelona Respiratory Network website (www.brn.cat/microbiome2016).

Challenges for different scientific disciplines

The bioinformatics view

The 16S rRNA gene has several variable regions that can be used for bacterial and archaea classification (*i.e.* taxonomy) [8–10]. Further, because its sequencing is fast and relatively inexpensive [11], it is often used to determine the composition, abundance and diversity of bacteria and archaea harboured in different ecosystems, such as the human respiratory tract [12–14]. However, this method has some important limitations. Firstly, as in any research activity, researchers must identify the right question and select the appropriate workflow from a range of available bioinformatics tools to address the question properly [15], because too many analyses can generate confusion and lead to loss of study focus. Secondly, appropriate control of the potential sources of variation in the study, including patient diversity, sampling methods, DNA extraction procedures, amplification and sequencing batches, is essential in microbiome research because they can all easily introduce unwanted variability and unexpected biases [16] (table 1). As discussed below, trying to keep these sources of variation as low as possible is the best strategy to overcome these hurdles. Thirdly, 16S rRNA gene sequencing does not provide information about viruses and fungi, or

Box 1 General terminology

Microbiota: microbial community membership associated with a defined habitat, such as the human body.

Microbiome: the genetic information (genomes) and inferred physico-chemical properties of the gene products of a microbiota.

Human microbiome: microbiome collectively found in internal and external habitats of the human body.

Metagenomics: shotgun random sequencing of total DNA in a sample, including DNA from host and microbe origin, which is analysed, organised and identified using sequence databases and computational tools.

16S ribosomal RNA (16S rRNA) gene: component of the 30S small subunit of prokaryotic ribosomes. It is used in reconstructing phylogenies owing to the extremely slow rate of evolution of this gene and the presence of both variable and constant regions allowing amplification.

Hypervariable region of the 16S rRNA gene: a DNA sequence that demonstrates diversity among different bacterial species.

16S rRNA gene analyses (or gene sequencing): a common amplicon sequencing method used to identify and compare bacteria present within a given sample. 16S rRNA gene sequencing is a well-established method for studying the phylogeny and taxonomy of samples from complex microbiomes or environments that are difficult or impossible to study.

Amplicon: DNA product of DNA amplification *via* PCR.

Shotgun sequencing: method for DNA sequencing in which DNA is fragmented into segments that are sequenced.

Dysbiosis: alteration of microbiota composition linked to perturbation of local ecological conditions, generally associated with impaired host–microbe interactions.

TABLE 1 Major sources of variability in microbiome studies

Sampling	DNA extraction	16s amplification and sequencing	Bioinformatics
Processing biases	Species bias due to different wall composition	Selection of regions to amplify	Thresholds for abundance
Constraints associated with type of sample	Batch effect [#]	Polymerase chain reaction and sequencing errors Adapter addition [¶] Batch effect	Alignment of sequences to databases Classification of sequences

[#]: batch effect refers to the bias introduced if not all samples are processed at the same time, in a single batch; [¶]: adapters are oligonucleotides that are ligated to the amplified DNA in order to do the sequencing. The efficiency of the ligation process can influence the sequencing results.

about their interactions with the bacterial microbiota, which need to be investigated using alternative approaches such as metagenomics and/or internal transcribed spacer sequencing. Finally, from a purely bioinformatics point of view, a number of issues related to the incompleteness of databases and methodological constraints discussed below (see Box 2 for current terminology) also need to be considered.

Database constraints

The existing 16S rRNA gene databases currently provide (partial or complete) gene sequences for more than 1.7 million bacteria and archaea [17], and are detailed enough to classify bacteria at different taxonomic levels, from phylum (high taxonomic level) to genus (low taxonomic level) (figure 1). Yet, these databases contain unresolved information for some sequences, so species-level identification is not attainable for some microorganisms [18]. It is also possible, owing to high levels of 16S sequence homology between species, that a sequence gives more than one hit with the same score in two or more different records in the database, indicating an inability to differentiate them. To resolve this situation, the “lowest common ancestor concept” is generally used [19]. Following this approach, the assignment of taxa is not given at the level of species, and reaches only the genus level for some bacteria. For example, this is the case for the *Streptococcus* genus, which is prevalent in the respiratory system and includes pathogenic bacterial species such as *S. pneumoniae* and commensals such as the viridans streptococci group. This limitation of species assignment obviously restrains the scope for identifying microorganisms ascribed to these genera. For all these reasons, bioinformatics tools used in microbiome research generally use a common approach to cluster sequencing reads at some level of similarity under the general term operational taxonomic units (OTUs). Thus, sequence similarities of at least 97% with the reference database of 16S rRNA sequences are generally acceptable to consider the identified OTUs as equivalent to the species level, or to the genus level when the similarity only attains 94%.

Methodological issues

As indicated above, the 16S rRNA gene has several variable regions (V1–V3 or V3–V5) that can be used for bacterial taxonomy purposes [3, 8, 9]. However, it is unclear which of them provides the best assessment of

Box 2 Bioinformatic terminology

OTU (operational taxonomic unit): cluster of microorganisms, grouped by DNA sequence similarity of a specific taxonomic marker gene, e.g. 16S rRNA. OTUs are used as proxies for microbial “species” at different taxonomic levels: phylum, class, order, family, genus and species. Sequence similarity is defined based on the similarity criteria; e.g. the sequencing reads with 97% similarity can be clustered together and represent a single OTU, and for some bacteria can attain the equivalence of the species level.

Diversity: the number and distribution of distinct OTUs in a sample or in the originating population. Thus, so-called alpha-diversity estimates describe the number of species (or similar metrics) in a single sample, while beta-diversity estimates describe the differences in species diversity between samples. A widely used diversity index is the Shannon–Wiener diversity index.

Relative abundance: how common or rare an OTU is relative to other OTUs in a community, measured as a percentage of the total number of OTUs in the population. Thus, OTU abundance is treated as a surrogate measure of bacterial species abundance.

Evenness: measure of the similarity of the relative abundances of the different OTUs in the population.

Taxon: group of one or more populations of an organism or organisms considered to form a unit.

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	<i>Escherichia</i>
Species	<i>Escherichia coli</i>

FIGURE 1 Taxonomic classification of *Escherichia coli*.

the respiratory microbiome. Moreover, it has been demonstrated that different sequencing platforms, including 454, Illumina HiSeq and MiSeq, can produce different results [16]. This is partially due to the specific variable region of the 16S gene used, the primers employed and the length of the amplicons produced by the different platforms. To reduce sequencing errors, longer reads are preferred [16]. An additional methodological problem is that the use of different algorithms, assumptions and parameters can lead to different results [13, 19, 20]. Therefore, it is important to be aware of these limitations and, if possible, use different sequencing and bioinformatics tools (*e.g.* marker gene, shotgun genome or transcriptome sequencing) to compare results obtained with different methods. Finally, it is worth saying that 16S sequencing provides qualitative but not quantitative microbiome information, and complementary methods such as quantitative PCR or digital PCR are recommended to complete the information attained through the analysis of the 16S rRNA gene.

Other bioinformatics challenges

Other bioinformatics challenges to consider include the following. First, most of the studies performed until now have estimated per taxon relative abundances based on the number of copies of 16S rRNA genes recovered in a sequence library [21]. Yet, variation in gene abundance can result from differences in the actual bacterial load or from the genomic copy number that a specific bacterial taxa is able to attain during the analytical procedure. The relative weight of these two factors on estimates of microbial community structure is unknown, but can be a source of systematic bias in studies using 16S rRNA sequencing. There are methods that correct for the copy number of 16S rRNA genes, but this correction is available for <5% of known bacterial species [22]. It is also worth noting that other genes like *cpn06* can also be used to infer bacterial community diversity [23]; thus, the possibility of using more than one gene should also be considered. Second, to compare results between studies performed in different laboratories it is recommended that mock communities are used, created *in vitro* with a predefined content of bacterial operons specific for the lung microbiome [24], but it may be more convenient to create consortia that would perform all the analyses in a single centre. Third, differences in DNA extraction [25] and PCR amplification methods can also introduce methodology-related variability [26].

The view from respiratory medicine

The microbiome in the healthy lung

The study of the normal human lung microbiome is still in its infancy, but it is clear now that healthy lungs harbour a phylogenetically diverse microbial community [2, 3, 27–31]. Results of published studies are somewhat limited by their small size and lack of longitudinal sampling but show that, in healthy subjects, Firmicutes, Bacteroidetes and Proteobacteria are the most frequently identified bacteria at the phylum level [32]. At the genus level, *Prevotella*, *Veillonella* and *Streptococcus* are the predominant microorganisms, with a minimal contribution from common pathogenic Proteobacteria including *Haemophilus* [32]. Healthy airways are challenging to sample because healthy subjects do not produce spontaneous sputum, so

sampling requires bronchoscopy, and repeating the endoscopic procedure in healthy individuals is cumbersome, limiting the possibility of having longitudinal data. However, recent studies that included bronchoscopic sampling of the proximal and distal bronchial tree have reported that the microbiota of the oropharynx, the bronchial tree and the alveolar surfaces have a similar composition in healthy individuals [29]. This similarity has been attributed to aspiration of oropharyngeal secretions during sleep [33–35]. This scenario may be altered in respiratory diseases, where perturbation of growth conditions in the bronchial tree and lung parenchyma promotes a shift in microbial community composition, with potentially pathogenic bacteria able to persist for longer periods of time [3, 29–31] (figure 2).

In any microbiome study contamination is a concern, and the potential contamination of lower airway samples by the oropharynx microbiota is a major issue to be specifically addressed in respiratory diseases [3]. The respiratory system lodges lower amounts of microorganisms than other human body surfaces, and low biomass samples such as those obtained by a protected specimen brush (PSB) or bronchoalveolar lavage (BAL) may not provide sufficient DNA, while the background signal from reagents may be misinterpreted as a real signal [36]. Thus, in order to discriminate signal from noise, proper technical controls are critically needed in sequence-based analyses of samples, particularly in respiratory samples, which may suffer from a dilution effect.

Information on the long-term effects of smoking on the respiratory microbiome of healthy subjects is scarce, and clearly needs research. Initial studies of the oropharynx microbiota in smokers have reported modifications in the microbial composition, affecting mainly the Firmicutes phylum and *Neisseria* species, that are important enough to be considered as dysbiosis [37], and a decrease in the relative abundance of Proteobacteria; these modifications do not revert after giving up smoking [38]. By contrast, studies of the respiratory microbiota in bronchial secretions have not identified significant differences between smokers and non-smokers [37], nor relevant changes in bacterial diversity after smoking cessation [39], suggesting that exposure to smoke results in proximal microbiome changes that are not reflected by corresponding downstream alterations in the bronchial tree, at least in the absence of respiratory disease. Differences in the oral microbiome of current *versus* former smokers with and without respiratory disease have not been properly assessed, however, and it is not currently possible to properly discern temporary dysbiosis caused by the exposure to irritants and acute injury from dysbiosis associated with chronic disease.

Chronic obstructive pulmonary disease

Bronchial colonisation by potentially pathogenic microorganisms has been well established in chronic obstructive pulmonary disease (COPD) by several previous studies [40, 41], but the direction of causality between this colonisation and airway inflammation, airflow limitation, and bronchial and lung parenchyma destruction remains unsettled. There is evidence of a relationship between the appearance of symptoms of exacerbation and the acquisition of new bacterial strains [40], but this change in the bacterial flora only partially justifies the appearance of exacerbations.

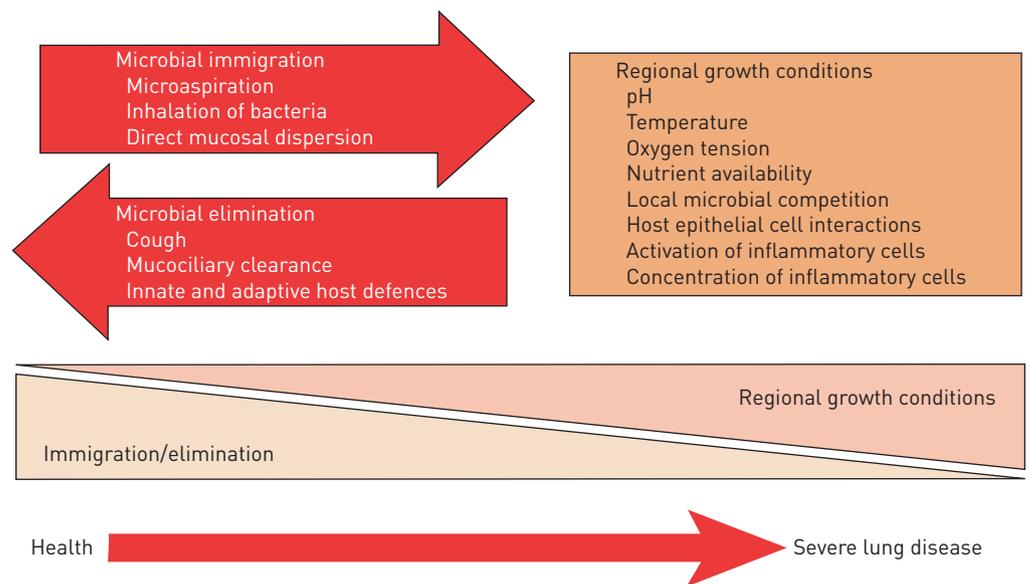


FIGURE 2 Key factors determining the respiratory microbiome: microbial immigration, microbial elimination and the relative reproduction rates of its members. In healthy subjects, the microbiome is determined mainly by immigration and elimination. In severe lung diseases, however, regional growth conditions are a main determinant of microbiome composition. Reproduced from [102] with permission.

In patients with clinically stable COPD, several studies have now reported a rich lung microbiome that is clearly different from that seen in healthy controls [2, 27, 30, 31, 42–46]. Common phyla in these patients are Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes, with *Pseudomonas*, *Streptococcus*, *Prevotella* and *Haemophilus* being common genera in these patients [2, 27].

Most data available from COPD come from samples obtained from biopsies [46], lung tissue explants [30], BAL or PSB [2, 27, 31, 46], and sputum [43–47]. Different sampling procedures target different regions of the respiratory system, however, and results have shown that sputum harbours microbial communities that are different from those in bronchoalveolar samples [46], and have confirmed that, in fact, bronchi and alveoli of patients with COPD contain a distinct microbiome [3] (see figure 3 in Dickson *et al.* [3]).

During exacerbations, some genera increase their relative abundance whereas others do not significantly change [42, 44, 48, 49]. In addition, exacerbations seem to be associated not only with over-representation of isolated genera, but also with collateral changes in microbiome composition as a whole, which in turn appear to be associated with increases in inflammation-related markers in BAL [41, 50]. Additionally, there seem to be interactions between viral infections and bacterial community composition, with increases in the relative abundance of Proteobacteria after experimental rhinovirus infection [48]. Similar interactions have been proposed between fungi and bacteria [51]. Furthermore, treatment during exacerbations influences the respiratory microbiome differently when based on antibiotics, which reduce bacterial abundance, mainly of Proteobacteria, *versus* oral steroids, which when administered systemically do not influence bacterial richness but favour an over-representation of specific taxa [40, 52].

Finally, several challenges need to be tackled before benefits from microbiome research in COPD can be meaningfully incorporated into clinical practice: 1) with regards the reported differences in the respiratory microbiome of distal and proximal bronchi, targeted by BAL and sputum respectively [46], meaningful thresholds need to be determined to identify clinically significant bacterial over-representations for all sample types; 2) the role of non-cultivable but potentially pathogenic microbes identified by microbiome studies is unclear and needs to be investigated; and 3) interactions between bacteria, viruses and fungi with the host need to be targeted.

All in all, despite these important hurdles, lung microbiome research has the potential to unravel new and relevant insights into COPD pathogenesis that may lead to better clinical management of COPD. Specifically, there is a clear need to understand the impact of current standard COPD treatments, particularly of inhaled corticosteroids, on the COPD airway microbiome, because these agents have been shown to reduce the frequency of exacerbations but, at the same time, to increase the risk of pneumonia, possibly through direct modulation of the airway microbiome. Eventually, changes in the microbiome may become important mechanisms (*i.e.* endotypes) underlying the different clinical presentations (*i.e.* phenotypes) of COPD.

Cystic fibrosis and bronchiectasis

Airway bacterial infection is central to our understanding of the pathophysiology of cystic fibrosis (CF) and (non-CF) bronchiectasis. Traditional culture-based microbiology techniques have revealed the importance of well-known pathogens such as *H. influenzae*, *P. aeruginosa* and *Moraxella catarrhalis* in bronchiectasis [53], and additionally *Staphylococcus aureus* and *Burkholderia cepacia* in CF [54]. Microbiome studies are moving our understanding of these two diseases forwards. For instance, previously unrecognised organisms are abundant in some patients, both in CF [55, 56] and in bronchiectasis [57, 58]. In addition, studies characterising the airway microbiome following antibiotic treatment have shown a remarkable resistance of bacterial communities to change over time in these patients [57, 59, 60]; antibiotic treatments primarily result in a reduction in bacterial diversity, but this effect disappears after some weeks, with the recovery of the previous microbial composition [57]. Overall bacterial diversity, measured using composite indices such as the Shannon–Wiener diversity index, has been linked to the level of airflow limitation present and other markers of disease severity both in CF and bronchiectasis. Additionally, an Australian randomised clinical trial in patients with non-CF bronchiectasis has shown that the relative abundance of potentially pathogenic microorganisms from the *Pseudomonas* genus increases in patients receiving chronic treatment with macrolides [60], but the extent to which the microbiome changes are attributable to the antibiotic regime is not known. The role of fungi, viruses and *Mycobacteria* (which are not identified by standard bacterial 16S rRNA sequencing) is unclear in both CF and bronchiectasis, and requires future research [61]. Likewise, other important questions that need to be examined in this clinical setting include the extent to which 16S rRNA gene sequencing provides useful clinical information beyond culture, the interactions with the host, the possibility to select antibiotic treatment based on microbiome profiles, the usefulness of microbiome results to evaluate therapeutic responses, the prognostic implications of microbiome analyses and the effect of antibiotics on the emergence of new pathogens. The ease with which sputum can be obtained in these patient populations facilitates large-scale studies in the coming years.

Interstitial lung diseases

Traditionally, interstitial lung diseases (ILD) have been considered to be non-infectious parenchymal lung diseases. However, the recent characterisation of the respiratory microbiome in idiopathic pulmonary fibrosis (IPF) has shown an over-representation of specific organisms such as *Streptococcus*, *Prevotella* and *Staphylococcus* in these patients as compared to healthy controls [62, 63] (figure 3). Whether or not they can drive disease progression is a hypothesis that merits future research [63].

The existence of acute exacerbations of IPF has been increasingly recognised as a major cause of mortality in these patients [64]. The exact pathogenesis of these episodes remains unclear, and current diagnostic criteria specifically require the exclusion of any infective trigger [65]. Despite this, there is evidence supporting an infectious hypothesis of IPF exacerbations: 1) a randomised controlled trial showed reduced mortality in patients who received prophylactic cotrimoxazole [66], 2) immunosuppression is associated with an increased rate of acute exacerbations [67], 3) a higher proportion of exacerbations occurs during the winter months, and 4) infectious episodes confer an identical mortality to non-infective exacerbations [63]. There is therefore great interest in using culture-independent molecular techniques to explore the role of infection in acute exacerbations of IPF, although the unpredictable nature of these events and difficulty in sampling have been limiting factors in addressing this topic.

Microbiome research in the entire range of different ILDs should establish 1) if there is any role at all of lung microbial composition in their occurrence and evolution; and 2) what the optimal sampling modality is in these patients, given that these parenchymal diseases may not be appropriately represented by bronchial samples such as sputum.

Lung transplantation

Owing to the long-term use of prophylactic and/or therapeutic immunosuppressive drugs and antibiotics, the lower airways of lung transplant recipients offer a special niche for the resident microbiota [68, 69]. In fact, alterations in local conditions during the first months post-transplant facilitate lower airway infections due to

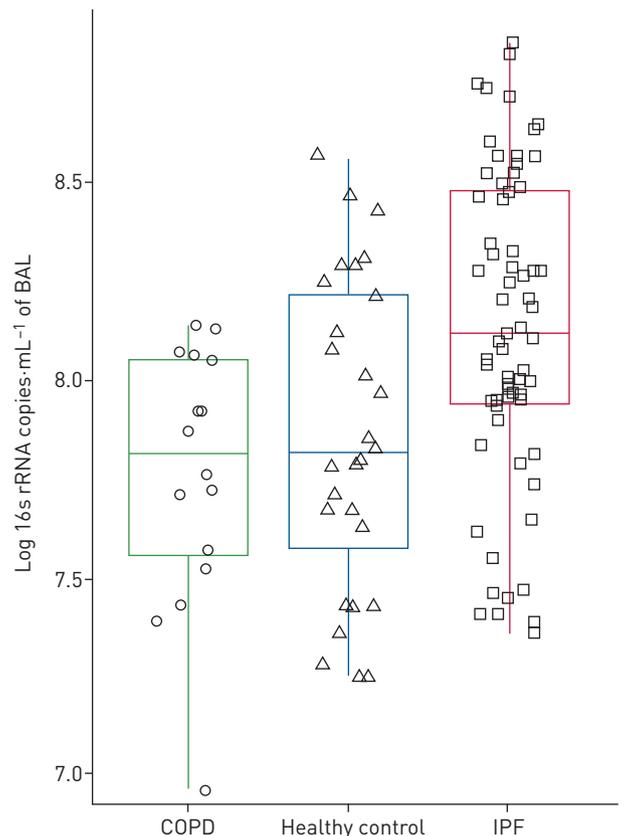


FIGURE 3 Bacterial load (16S copy number · mL⁻¹ of bronchoalveolar lavage (BAL)) in patients with idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD) and healthy controls. Patients with IPF (red; n=64) had a significantly higher bacterial burden than subjects with COPD (green; n=17) and the healthy control subjects (blue; n=27) ($p=0.006$ and $p=0.0007$, respectively). The box signifies the 25th and 75th percentiles, and the median is represented by a short line within the box. Reproduced from [53] with permission.

opportunistic bacterial pathogens. A blunted inflammatory status commonly prevails between 6 and 12 months post-transplant, in association with a strong predominance of bacteria typically found in the oropharyngeal microbiota [70]. Modifications in the respiratory microbiota composition in the lung transplantation setting are strong enough to be considered as dysbiosis, and are manifested through the over-representation of specific OTUs, including those listed below, that have been related to the persistence of abnormal underlying host inflammatory profiles [70, 71]. Furthermore, the onset of bronchiolitis obliterans syndrome following transplantation has also been linked to host–microbe interactions, through pathogen-driven inflammatory triggers and/or impaired host innate responses affecting bacterial clearance [72, 73].

Studies using culture-independent techniques that identified microbiota dysbiosis in patients with lung transplants reported a frequent clear-cut predominance of Proteobacteria and/or Firmicutes, linked to microorganisms from the *Pseudomonas* and *Staphylococcus* genera [68, 74], and Burkholderiaceae family [75]. These bacteria, which may represent over 70% of the BAL microbial community, are typically associated with a pro-inflammatory response, whereas an over-representation of similar magnitude of Bacteroidetes, mostly due to the abundance of *Prevotella*, was instead linked to a remodelling host gene expression profile [70]. These findings suggest that microbiome–host interactions influence innate immune processes within the transplanted lung. Future research should try to relate these patterns to long-term allograft outcome and the risk of transplant rejection occurrence.

Lessons from other human organ systems: the gut

Gut microbiome research has pioneered the field of microbiome research and is far more advanced than that of respiratory microbiome. First, it is now using next-generation sequencing techniques, which allow the understanding of microbial communities in greater depth through the study of microbial genes or full genomes [76], and metatranscriptomics, which include RNA sequencing (see the terminology in Box 3). Second, initiatives like the Human Microbiome [77] and the MetaHIT [78] projects, sponsored by the National Institutes of Health (USA) and the European Commission, respectively, have allowed a deep characterisation of the human gut microbiome in health and disease states. As a result, we now know that the human gastrointestinal (GI) tract harbours one of the most complex and abundant existing microbial communities of more than 100 trillion microorganisms, with the number of microbial genes exceeding by about 100-fold the number of human GI cells. Although stable across ages, the composition and functions of the intestinal microbiome is influenced by a number of factors, including genetics and exposures at birth related to delivery, age, geographic location, diet, smoking and medical treatments [79]. Third, while there are also many potential sources of variability that can significantly impact the results of GI microbiota studies, a global effort has been made to define best practices and protocols to compare different GI microbiota studies, meta-analyse them and extract new knowledge. The protocols of this effort, the International Human Microbiome Standards Project, are available online (www.microbiome-standards.org). Fourth, the gut microbiota not only influences the GI tract, it can also affect many functions of the body, ranging from processing and harvesting of nutrients from our diets, to the shaping of innate and adaptive immune system responses [80, 81]. Hence, GI microbiota changes can favour the development of GI as well as non-GI diseases. For example, a vast body of literature now links functional and metabolic GI disorders, such as inflammatory bowel disease, irritable bowel syndrome or obesity, with gut microbiome alterations [82–85], but there also reports of a relationship between changes in the gut microbiome and neurological disorders (e.g. autism) [86–89] and respiratory diseases (such as the acute respiratory distress syndrome occurring in patients with septic shock [90]). Fifth, the HIV epidemic has taught us that homosexual men often have a distinct composition in their faecal microbiota, with increased microbial richness and diversity, as well as enrichment in the *Prevotella* enterotype, independent of their HIV status [91]. HIV-1 infection is associated with reduced bacterial richness, particularly in subjects with suboptimal CD4+ T cell counts under antiretroviral therapy [91]. Finally, interventions designed to modify the composition of the gut microbiome have been successful in specific GI diseases. Faecal microbiota transplantation is becoming increasingly accepted as an effective and safe intervention in patients with *Clostridium difficile* infection, and different centres have reported success rates >90% with this treatment [92]. This approach is much more complicated in inflammatory bowel disease, where faecal transplant has success rates of around 13% [93]. The effects of the bacterial modifications of the gut microbiota on the respiratory tract microbiome of

Box 3 Other systems terminology

International Human Microbiome Standards: standard operating procedures designed to optimise data quality and comparability in the human microbiome field.

Faecal transplantation: process of transplantation of faecal bacteria from a healthy individual into a recipient.

healthy subjects and patients with varied respiratory diseases, as well as potential indirect effects *via* alterations in the host immune response (and their response to faecal transplant) to date have not been properly addressed. Current knowledge, including early-life beneficial and detrimental alterations of the gut microbiota, and its relationships with allergic respiratory diseases, have been recently reviewed [94], and the gut–lung axis now offers a wide range of research possibilities, as discussed below.

Workshop limitations and further reading

The present manuscript is a report of a workshop on which some aspects that deserve comment were not covered. Several investigators have addressed the role of the microbiome in asthma and paediatric diseases other than CF, a research field that has recently been reviewed [95–98]. These reviews interestingly describe mouse and human data of the lung–gut axis on asthma development. Similarly, the role of anaerobic bacteria in respiratory disease has been only marginally addressed in diseases such as CF and bronchiectasis to date [57, 99–101] and needs focused research.

Future respiratory microbiome research

From the above discussion, participants in the workshop agreed on the following nine specific aspects that need to be specifically addressed by future respiratory microbiome research:

1. **Normality patterns:** Studies performed in healthy subjects so far have clearly demonstrated that there is a rich microbiota in the respiratory system that includes microorganisms from the Firmicutes, Bacteroidetes and Proteobacteria phyla, and displays a close similarity to that of the oropharyngeal microbiota. Normality patterns for viruses and fungi still need to be defined, however. The microbial composition of the respiratory microbiota changes in chronic respiratory diseases, but the timing and the distribution of these changes are only partially known.
2. **Diversity in sampling procedures:** There is a wide consensus that the best sample procedure depends on the question being addressed. Sputum may be an appropriate approach for the study of respiratory diseases that have a significant bronchial component, considering that it can be obtained from a wide range of patients and does not require invasive procedures, but more reliable information on the peripheral bronchial tree and alveolar surfaces requires invasive samples (*i.e.* BAL, PSB, bronchial or lung biopsies). Similarly, in GI tract research, faeces are now collected for large studies and local biopsies are used to answer specific questions in a restricted number of patients. In any case, these measurements still need to be paralleled by conventional microbiological studies because, although sequencing provides a general picture of the composition of the bacterial community, microbiological cultures provide clinically meaningful information on the role of respiratory pathogens such as *Haemophilus* and *Pseudomonas* in disease, which still do not have an equivalent in microbiome analyses.
3. **Standardisation:** There is a pressing need to standardise protocols to be used to analyse the respiratory microbiome, including sampling, processing and bioinformatics methodologies. The creation of consortia and networks for research on this topic would facilitate this standardisation and, as a result, the possibility of sharing results from different cohorts.
4. **Non-cultivable and/or non-pathogenic bacteria:** 16S rRNA gene analyses have shown high relative abundance and specific patterns of non-cultivable microorganisms (with a general over-representation of Proteobacteria) in bronchial and lung samples obtained from patients with COPD, IPF, CF and bronchiectasis. The role of specific species previously considered non-pathogenic needs to be addressed in these different clinical conditions.
5. **Loss of diversity:** Loss of diversity has been related to disease severity in COPD, IPF and CF, and it has also been described during exacerbations of these diseases. A similar observation has been reported in the gut, suggesting that a general pattern of a decrease in the diversity of the microbial composition associated with the over-representation of specific OTUs may occur in human diseases, but the temporal dynamics of these microbial changes are widely unknown. What drives this loss of bacterial diversity, including the impact of interspecies competition, antibiotic exposure and host immune responses, must be defined.
6. **Interactions with the host:** Data on microbiome–host interactions is incomplete in gut diseases and almost non-existent in respiratory diseases. Future studies should address both the local and systemic impact of microbial communities, because important remote effects can be exerted through the release of mediators in the bloodstream. Hence, dissecting the intricate interplay of host–microbe interactions in different body sites, such as the lung, gut and skin, represents a major challenge in future microbiome research but has the potential to help clarify the determinants of progression in several chronic respiratory diseases. To properly assess this point, new studies should include research on the diversity of the microbiome in the same host at several sites; have a longitudinal dimension; assess the local and systemic immunity of the host; and, finally should prove the effects of microbiome patterns on the pathogenesis of respiratory diseases through microbiome transplantation in animal models.

7. **Bacterial RNA and metagenomics:** After 16S rRNA gene analysis, a new stage in the study of the microbiome is beginning with DNA shotgun sequencing and RNA analysis. These techniques need to be implemented in the study of the respiratory microbiome because they will provide functional information, which is absent in 16S rRNA gene analyses. Furthermore, 16S rRNA gene cannot differentiate between living and dead bacteria, and how long DNA from dead bacteria persists in respiratory samples is not known.
8. **Viruses and fungi:** The role of viruses, including the vast number of phages that infect bacteria, and fungi in respiratory health and disease cannot be targeted through 16S rRNA gene analyses, and needs investigation. Interactions between viruses, fungi and bacteria have been only marginally assessed so far, but preliminary results have shown well-defined effects of non-bacterial microbiota on Proteobacteria abundance.
9. **Interventions:** Bacterial supplementation and modulation of the microbiota through probiotics and equivalents has not yet been explored in respiratory diseases, but it is a potentially fruitful research field. Whether probiotics directly targeting the lung parenchyma, or restoring normal upper airway or gut microbiota, can produce beneficial effects in respiratory diseases remains to be determined.

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