Review

Lower Respiratory Tract Microbiome

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ABSTRACT
Given the fact that the hypothesis of respiratory microbe sterility is being degraded, researchers are currently studying healthy lung microbiota homeostasis as well as its disturbance in case of illness. Taking into consideration the thorough understanding of diseases that gut microbiota studies offered, one cannot but anticipate that respiratory microbiome research would reveal further insight to the pathogenesis of lung disorders but mostly to healthy respiratory physiology. In this article, we review published studies with a view to summarizing important terms and definitions in human microbiome studies, obstacles in lung microbiota research, microbial diversity of lower respiratory tract in health state and major lung diseases, including chronic obstructive pulmonary disease (COPD), asthma, interstitial lung disease (ILD), lung cancer and cystic fibrosis.
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INTRODUCTION

Microorganisms are well known to occupy all human body sites, most of them inhabiting the gastrointestinal tract[1], though different communities were shown to also reside on skin[2], vagina[3], the oral cavity[4] and recently lower respiratory tract[5,6]. To characterize microbial communities colonizing multiple body sites and highlight their disturbance in case of disease, National Institute of Health (NIH) funded the Human Microbiome Project. Surprisingly, lower respiratory tract was not included as a site of interest[7]. Due to the increasing attention shown by researchers, the National Heart Lung and Blood Institute currently supports the so-called Lung HIV Microbiome Project, a collaborative project, still in progress, aiming to outline lung microbiome and shed light to changes observed in lung disease[39].

MODERN TECHNIQUES TO STUDY THE MICROBIOME

Terms and Definitions

To clarify the terms herein used it would be useful to provide readers with the following definitions. ***Microbiota*** makes reference to all the living

microorganisms inhabiting a particular ecologic niche, such as the respiratory or gastrointestinal tract. The term ***microbiome*** refers to the genome of microorganisms entire population, including their metabolites[8]. Since the beginnings of microbiology, in the 17th century, reseachers compared human microbiota, of different habitats (oral-feces) and individuals to prove differences in health and disease state[9]. Recently, it came as a surprise to them that the human microbiota numbers 10 to 100 trillion cells, more than 10 times the human somatic and germ cells[10].

Since the introduction of plating techniques by Robert Koch, in late 1880's, and intensively during the past decades, ***culture-based techniques*** have been widely used for bacteria identification in a plethora of biological fluids samples, including sputum and bronchoalveolar lavage (BAL). Samples have been plated on specific media, shown to be suitable for each microorganism growth. Therefore, microbiota has been identified according to the medium allowing growth, the unique structural features of colonies and the metabolites produced or depleted accordingly[11]. Traditionally, phenotypic traits (phenotypic fingerprinting), such as optimal growth temperature or medium, have been used to identify different strains (strain-typing). These methods are highly dependent on the researcher's ability to simulate specific growth conditions *in vitro*[12] and consequently can be difficult to standarize. To overcome this, researchers introduced genotypic fingerprinting. Strains populations are recognized and distinguished by phenotypically similar ones, usually using special restriction enzymes to digest their DNA and genetic probes to label these unique for each strain fragments[13,14].

Taking into consideration that traditional culture-dependent techniques have been reported to identify microbiological etiology in only 25% of patients diagnosed with pneumonia in ward and ICU[15], the need for introduction of novel methods in pathogen identification was raised into a burning issue.

Over the last 20 years ***culture-independent techniques*** have been put into practice in order to identify microbial communities in different niches of human body (such as gut, lower respiratory tract, skin etc.). The introduction of Polymerase Chain Reaction (PCR), microarrays and metagenomics led to deeper knowledge of microbiota characteristics in healthy persons and patients suffering from diverse disorders (Figure 1).

Making a breakthrough in the 1980's Woese and coworkers showed that the gene coding for 16S rRNA can depict phylogenetic relationships among bacterial strains[16], since it shows remarkably high conservation, a

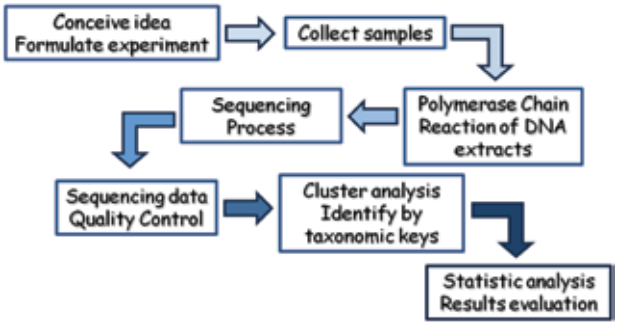

FIGURE 1. Flowchart summarizing steps of a microbiome study, from concept to results.

fact probably due to the significance of this component of the small subunit of ribosomes for proper cell function[17]. 16S rRNA gene is around only 1.5kb long thus allowing for quick sequencing outcomes[18] and consists of highly conserved regions alternating with 9 hypervariable regions (V1-V9). Small sequences within these hypervariable regions were shown to differentiate among bacterial species[19]. Sequence analysis and comparison of accurate sequences with those available in verified databases have allowed identification of mycobacteria, who traditionally need a long time to be cultured[20], and unculturable bacteria, such as *Treponema pallidum*[21].

Until recently researchers have been widely using the Sanger sequencing technique, to accurately determine DNA sequences[22]. This method is based on elongation of the primer of interest using a DNA polymerase, normal deoxynucleotides (dNTPs) and specially modified and flagged dideoxynucleotides (ddNTPs). The latter lack 3'-OH group, which is indispensable for the formation of the phosphodiester bond, holding the next nucleotide closely attached to the elongating chain. Each time such a modified molecule is attached DNA chain elongation ceases. After many rounds of DNA extention, using PCR, and separation through (gel or capillary) electrophoresis, sequence is determined identifying either the radio-labelled or dye-labelled chain terminators[23,24].

To overcome the major disadvantage of conventional Sanger method, which is poor throughput, next generation sequencing (NGS) techniques have been recently introduced. A plethora of different NGS platforms, based on various sequencing technologies, can carry out multiple sequencing reactions, thus sharply increasing output. Furthermore, these techniques provide many copies of shorter sequencing reads (less than 400 base pairs) coming from single DNA molecules, unlike Sanger sequencing

technique which required PCR amplified samples while giving out single long reads. In this way not only accurate sequencing results are delivered but mutations in small cells subpopulations can be recognized[25,26].

Lung microbiome analysis

In terms of lung microbiome research, the use of real-time quantitative PCR in sputum samples raised the percentage of successful microbiological agent identification up to 67% of pneumonia cases. This was further increased (87%) when full sampling was performed (sputum, blood and nasopharyngeal secretion samples)[27].

Culture-independent microbiological techniques have been recently used to analyse lung microbiota in bronchoalveolar lavage samples obtained through bronchoscopy. According to Hogan and co-workers this technique gives the chance to examine differences in microbiota among separate lung regions, even though statistically important difference has not yet been reported[28].

One of the main challenges shared among all the microbiome studies is the choice of primers used to recognize microbial diversity. Most studies use the V1-V3 and/or V3-V5 hypervariable regions of 16S rRNA gene to prove presence of bacterial taxa even though these regions were shown to sporadically give inconsequent results, especially as far as sub-genus operational taxonomic units levels are concerned[29]. Results were thus shown to be affected by the specific gene region targeted by pyrosequencing technique, thus making comparisons among studies insecure[30].

Procedure obstacles

Studying lung microbiome particularly, researchers had to deal with samples contamination coming from upper respiratory tract, since bronchoscope can drift a plethora of microbial factors passing through oropharynx. Taking into consideration the continuity of microorganisms found along the respiratory tract[30], a variety of methods have been proposed in order to reveal genuine lung microbiota. These include use of two equipments, with the first one being used to take supraglotic samples for comparison, and design of specific single-sided outlier tests, in order to identify bacteria replicating in lower repiratory tract notwithstanding oropharyngeal background[30,31].

Due to very low levels of microbiota reported in lower respiratory tract and great sensitivity of PCR in positive detection, sterile bronchoscope washes samples as well as reagent controls must always be included in studies to support reliable results[32].

It is also of great importance to mention that 16S rRNA gene sequencing technique, as well as other DNA-based methods, are proved to overrate lung bacterial burden, since they can discern no difference between viable and non-viable microorganisms. Further supporting our knowledge on the robust immune response of lower respiratory tract against bacteria[33], Pezzulo and co-workers showed that in pigs BAL fluids 63% of bacterial DNA retrieved was DNAse I sensitive, meaning coming from dead bacteria. It is worth noting that even if bacterial burden was shown to drop in BAL samples treated with DNAse I, bacterial diversity remained unaffected[34]. To shed further light to this, Venkataraman and co-workers, reported opposing results since they managed to cultivate 61% of species, identified with 16s rRNA gene sequencing methods in healthy human BAL samples, applying a variety of media and incubation conditions[35].

HEALTHY LOWER RESPIRATORY TRACT MICROBIOME

According to Dickson and Huffnagle it is the dynamic equilibrium of three main factors that affects the lung microbiome synthesis in health state or illness. The two opposing factors, playing the major role in healthy lung microbiome composition are **microbial immigration** from upper to lower respiratory tract parts and **microbial excretion**, usually achieved through cough, mucociliary clearance and host immune defense. Microbial movement along respiratory tract can be due to unintentional/subclinical aspiration of small quantities of fluids or secretions, bacteria carried by air flow and direct spreading due to airways mucosal continuity. Gastric reflux has also been suggested as a way to further repopulate the indigenous bacterial population of lower respiratory tract[36]. The third factor, reported to be altered in case of disease and thus to affect microbiome, are **growth conditions** as these are locally determined by temperature, ph, oxygen and nutrients abundance, host defense and inflammation activity[37] (Figure 2).

Bacterial populations

A plethora of studies describing the pulmonary microbiota of healthy individuals have been published. Due to small size of samples, inconsistency of methodology and lack of longitude these should be considered with caution, though being indicative[30,38,39]. In terms of phylum rank-

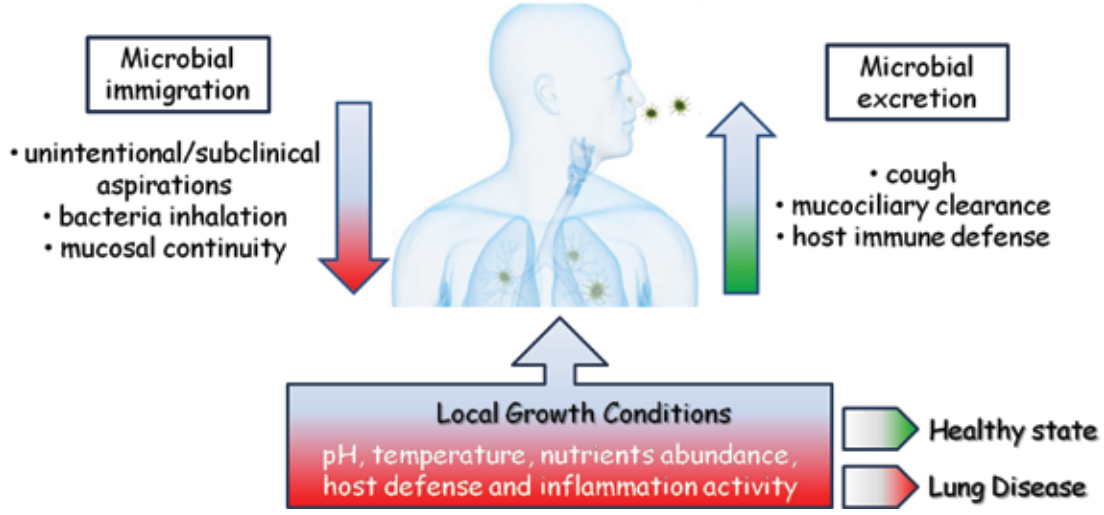

FIGURE 2. Three main factors affect the composition of lung microbial communities thus determining health or disease state of lower respiratory tract.

ing *Bacteroides, Firmicutes* and *Proteobacteria* have been systematically identified in healthy lungs using culture-independent microbiological techniques. *Streptococcus, Pseudomonas, Prevotella and Veillonella* were the genera identified in most controls in several studies comparing lung microbiome in BAL samples between diseased and healthy lungs[38-40].

Upper and Lower Respiratory Tract Continuity

Same bacterial taxa were recognized in mouth washes and BAL samples retrieved from healthy individuals[30]. It is worth noting that Morris and co-workers underline the presence of members of *Enterobacteriaceae and Pasteurellaceae* (mainly belonging to the genus *Heamophilus)* families. These particular populations were shown to exist in significantly larger quantities compared to neutral model, given the fact that they come from upper airways, thus showing that lung microbiota significantly differs from mouth bacteria[39]. Further enhancing results of Lozupone et al. and Charlson et al., the aforementioned study identified *Tropheryma whippleii* in healthy individuals BAL samples, even though this was not detected in their oropharynx samples[30,39,41]. To explain this phenomenon, Segal and Blaser suggested either the microaspirations scenario or this of hematogeneous spreading, thus implying that lung is an ecological niche for this specific bacterium[5]. In the same study, differences were reported between healthy smokers and non-smokers lung microbiota.

All in all, differing from gastrointestinal tract, it seems that respiratory tract has largely homogenous microbiota, scaling down in biomass as moving forward the respiratory system[30].

Transplanted lungs

Transplants were inhabited by a greater variety of different bacteria, as proven by bacterial sequences identified, compared to healthy controls. These were in the majority Proteobacteria, whereas in healthy lungs *Proteobacteria* (class *Gammaproteobacteria*) and *Firmicutes* were predominant[42].

Variations:

a. Geographical

Given the fact that geographical differences have been reported in the case of gut microbiota in healthy controls[43] and that as already mentioned temperature, oxygen, ph and nutrients presence play an important role in microbiota growth, it would be reasonable to expect that lung microbiome would be altered according to climate. Interestingly 19 pairs of sputum samples coming from patients diagnosed with cystic fibrosis from two centers (U.K. and U.S.) when analyzed using culture-independent techniques revealed significant heterogeneousness between the groups as far as the bacterial populations inhabiting lower respiratory tract[44]. No data is available comparing lung microbiome in healthy controls living across the world.

b. Spatial

Taking into consideration that microbial growth conditions (such as temperature, ph, oxygen tention) are well known to vary among different regions of healthy lungs[44,45], Dickson and co-workers examined whether lung microbiome varies accordingly. They showed that there is no significant spatial variation in healthy individual's lung microbiota, thus proving that BAL results coming from a discrete lung segment can be representative of the individual's microbiota, if healthy[47]. In contrast, lung microbiota was shown to differentiate among segments in severe COPD[38] and CF patients[48], thus raising the matter of unsuccessful infections treatment based in BAL samples in specific lung segments in such cases.

DISEASED LOWER RESPIRATORY TRACT MICROBIOME

a. COPD

Since early research times, COPD has been thought to be characterized by chronic inflammation[49], a situation partly induced by successive infections. This excessive inflammatory response is characterized by T-lymphocytes and macrophages penetration in the bronchial mucosa[50] and has been proven to be precipitated by bacterial causes[51].

Leading the way in COPD lung microbiome research based on culture-independent techniques Hilty and co-workers showed that COPD patients lower airways exhibited a statistically important decrease in *Bacteroidetes* (specifically *Prevotella* spp.) and a reverse increase in *Proteobacteria* phylum (particularly *Haemophilus* spp.), thus proving for the first time an alteration in COPD lung microbiota[52]. Interestingly, it was shown that no important differences exist in terms of quantitative results, though bacterial diversity was reported to be significantly diminished in patients diagnosed with moderate to severe COPD. Their BAL samples were highly abundant in *Prevotella*, *Pseudomonas*, *Streptococcus* and *Heamophilus*, genera which were though present in healthy controls too[38]. Sze and co-workers highlighted an important increase of *Firmicutes* phylum, due to higher burden of *Lactobacillus* genum, in severe COPD patients which could be though attributed to the fact that the lung tissue samples studied were mainly parenchymal[53]. Later, Zakharkina and researchers, enriched our knowledge about the core microbiota identified in lower respiratory tract of COPD patients, publishing that *Moraxella, Curvibacter* and *Corynobacterium* are some of the genera shown to characterize COPD state. Importantly, they correlated the presence of *P.Aeruginosa* with a significant decrease in microbiome diversity identified in COPD patients BAL samples[54].

It is worth noting that microbiota of COPD patients clustered not according to disease stage but according to the use of inhaled corticosteroids and other bronchodialators, a fact attributed to their interference with immune response to lung microbiota[55].

b. Asthma

It is well-known that asthma is another major pulmonary disease characterized by chronic inflammation, either precipitated by external stimuli (allergens) or microbial communities causing acute infection thus triggering an asthma exacerbation[56]. Early studies based on traditional culture-techniques or serologic testing implied chronic colonization or acute infection by *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* is associated with asthma beginning in adulthood[57,58]. *C. pneumoniae* were later proved by PCR techniques to be highly present in induced sputum, BAL and endobronchial biopsies of asthmatics compared to healthy controls[59,60].

Bisgaard and co-workers in 2010 showed that during exacerbations lower respiratory tract of asthmatic young children is inhabited by *Moraxella catarrhalis, Haemophilus influenzae, or Streptococcus pneumoniae*[61]. This is in accordance with previous study showing that early colonization of neonates hypopharynx by these species is associated with asthma beginning by the age of 5 and is a putative predictive marker for early-life wheeze, asthma and atopy[62]. Later studies based on sequence analysis by PCR revealed that nasal colonization by *Moraxella catarrhalis* and *Streptococcus pneumoniae* correlates highly with severe asthma exacerbations caused by rhinovirus[63].

Generally, adult asthmatics sputum samples showed greater variety in terms of bacteria than healthy controls, as well as a rich profusion of *Proteobacteria*[65]. Specifically, adults suffering from severe asthma had sputum samples rich in *Actinobacteria* and *Klebsiella* species, whereas those showing signs of moderate asthma had sputum samples abundant in *Proteobacteria* phylum[64]. Interestingly, *Proteobacteria* were also highly abundant in induced sputum samples of patients with mild asthma, who were not under treatment with corticosteroids, thus suggesting that this

microbiota alteration may be an inherent characteristic of asthma and not a result of immunomodulatory therapy[65].

c. Interstitial Lung Disease (ILD)

An alteration of lung microbiota is anticipated to be also present in the case of patients with ILD since a dysregulation in immune response and excessive inflammatory response is proven in lower respiratory tract of such patients[66]. Surprisingly, only recently Garzoni and collaborators using culture-independent techniques showed that lung microbiota of patients diagnosed with idiopathic interstitial pneumonia, non-idiopathic interstitial pneumonia and sarcoidosis is comparable with that of healthy individuals[67]. Earlier researchers reported the presence of *Haemophilus influenza* in BAL samples coming from patients with different interstitial lung diseases and a putative antagonistic relation between *Pneumocystis jirovecii* and bacteria inhabiting their lower respiratory tract[68].

As far as idiopathic pulmonary fibrosis (IPF) is particularly concerned, it was shown that specific genera, including *Staphylococcus* and *Streptococcus*, were more abundant in progressive disease rather than stable IPF state[69]. In more details, IPF patients were reported to have double the bacterial burden of healthy controls and specifically *Haemophilus*, *Streptococcus*, *Neisseria*, and *Veillonella* species. Furthermore this bacterial abundance was shown to be predictive of progressive lung dysfunction and death, shedding light to the pathogenesis of the disease[70].

d. Lung Cancer

Keeping in mind the proven association between the risk for several types of cancer and the presence of specific bacteria in human body niches (such as *Helicobacter pylori* and stomach cancer) researchers are currently studying a putative disturbance of lung microbiota in patients diagnosed with lung cancer and the impact of changes in lower respiratory tract microbiome in carcinogenesis[71]. Supporting this association, the meta-analysis of Brenner and co-workers revealed a pooled relative risk of 1.76 for patients with *Mycobacterium tuberculosis* infection to show later lung cancer[72]. A restricted study concerning females , with no smoking history who were diagnosed with lung cancer, showed a significant microbiome disturbance in sputum, which surprisingly was not recognized in oral washes samples. Their sputum samples showed higher abundance in *Streptococcus*, *Granulicatella* and *Abiotrophia* genera when compared to healthy controls. Given that these bacteria are well known to cause infections of nervous system, higher and lower respiratory tract and chronic vascular inflammation[73], and that a major pathway in carcinogenesis involves inflammation[74], these results may suggest a new role of microbiota in lung cancer pathogenesis.

A first study involving diagnostic endoscopic sampling of single pulmonary nodule has been published, showing taxonomic differences between the microbiomes of benign and malignant lesions sampled. Specifically, *Staphylococcus aureus*, *Pseudomonas* and *Haemophilus* species were more abundant in malignant cases, while no differences between peri-lesional and peripheral (coming from other lung segments) samples was reported[75]. To support these findings, Yu and co-workers in a recent publication showed that there is significant difference in microbial diversity between malignant and non-malignant lung tissue samples from cancer patients. Non-malignant tissues in paired samples showed higher microbial abundance. Specifically, non-malignant tissues from patients with advanced disease (stages IIIB, IV) were highly abundant in *Thermus* genera, whereas those from patients with metastatic disease were highly inhabited by *Legionella*. These results raised the issue of whether microbiota plays a role in tumorigenesis or the disease influences the microbiota of neighboring lung areas[76].

e. Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder characterized by mutations of the gene coding for cystic fibrosis transmembrane regulator (CFTR) protein, which plays an important role in ion transport (chloride, thiocyanate, bicarbonate) across epithelial surfaces[77]. Disruption of the lungs mucus layering leads to recurrent respiratory infections, chronic inflammation, progressive airway obstruction, damage of lung parenchyma and eventually death[78].

Since early times, culture-based techniques have been used to identify the microbiota inhabiting the lower respiratory tract thus establishing the main views of CF microbiology and treatment options for CF respiratory infections. Apart from *Pseudomonas aeruginosa*[79], Haemophilus influenzae[80] and *Burkholderia cepacia*[81] being major pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) has been isolated from CF patients and associated with higher risk of death[82].

During the last decade studies based on culture-

independent techniques proved the polymicrobial aspect of CF lower respiratory tract microbiota. *A. fumigatus*, S*tenotrophomonas maltophilia*[83], *M. avium-intracellulare* complex (MAC), *M. abscessus* complex (MABSC)[84], Achromobacter spp.[85] and Streptococcus milleri/anginosus group[86] have been added to the list of microbes inhabiting CF patients lung. Rogers and co-workers proved that CF lung microbiota is characterized both by high complexity and large quantities of bacterial species[87]. Recently, anaerobic bacteria were shown to be part of both "healthy" and "infected" CF lungs microbiota. Interestingly, higher airways inflammation and lower lung function is associated with reduced anaerobic load, a finding opposing the fact that anaerobes were shown to produce mediators of virulence, causing inflammation and acting synergistically with other putative pathogens[88].

In terms of disease progression it is well established that microbiota diversity reduces from early years to older ages. Coburn and co-workers showed that lower bacterial diversity correlates with deteriorating lung function, both forming a plateau at the age of 25[89]. This decreasing diversity, which was recently shown to be great among individuals[90], has been primarily attributed to the use of antibiotics. Interestingly, during disease exacerbations an alteration of the bacterial burden and diversity has never been proved[91], thus raising exacerbations to events of "intrapulmonary spread of infections"[92] and opening up new horizons to CF treatment.

IMPLICATIONS

It is now common knowledge that gut microbiota, which has been the target of intense study during the past decades, was shown to interact in a mutual way with host immune system. Importantly gut microbial communities were reported to be involved in pivotal signaling, thus boosting maturation of host immune cells[93] and therefore protecting from infections. According to the "hygiene hypothesis"[95] in early life years harmless pathogens such as, helminths, saprophytic mycobacteria, bifidobacteria and lactobacilli, induce abberant production of T regulatory cells, suppressing function of T effector cells, thus indirectly playing a protective role against inflammatory bowel disease and establishing immune tolerance[94,95].

In the same context, it has been shown that whereas farm and pet exposure during early infancy reduces the risk of atopy and asthma in coming years[96], the colonization of lower respiratory system of neonates by *S. pneumoniae* and/or *H. influenzae* and/or *M. catarrhalis* can lead to childhood asthma[62]. Further studies on healthy lung microbiota could give answers on what defines healthy microbial contact and shed light on the topic of healthy lung immune response and how this is disturbed in case of disease.

Furthermore, taking into consideration that in the case of gut microbiota three distinct types, so-called "enterotypes"[97], have been suggested as three main clusters of all human gut microbiomes, it would be of researchers interest to find out if this applies for lung microbiome too. Putative "pulmotypes" could help researchers classify these highly dimensional microbial communities into easily manipulated groups. Importantly, if human lung microbiome could be classified in distinct groups, individualized therapies, as well as diagnostic tools, could be designed for diseases already correlated with disturbed microbiota[98].

Recently lower respiratory microbiome has been extensively studied in a plethora of diseased lung states, including COPD[99], asthma[100], CF[90], ILD[67] and lung cancer[71]. These studies have shed light to the pathogenesis of the diseases, revealing unknown host to microbe and microbe to microbe interactions thus loading numerous quarrels to the quiver of treatments for clinicians to use. Nevertheless, it is the thorough knowledge of healthy lower respiratory tract microbiota that would allow further understanding on lungs physiology and reveal putative pathogens still hiding under the mask of healthy lung microbiota variation.

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