Defining the baseline of pulmonary microbiota in healthy populations and influencing factors

Zhuoning Tang 1, Sen Yang 2, Zilong He 3,*

1 West China School of Public Health, Sichuan University, Sichuan, China
2 West China School of Basic Medical Sciences & Forensic Medicine, Sichuan, China
3 School of Engineering Medicine, Beihang University, Beijing, China

* Corresponding author: hezilong@buaa.edu.cn

Abstract. Lung microbiota and lung diseases have already received increasing attention. However, the lung microbiota lacks a unified healthy baseline. In this review, we collect the healthy pulmonary microbial composition based on the data of existing relevant studies. Subsequently, we discuss and analyze the three aspects of bacterial, fungus and viral at the phylum and genus levels, as well as influence factors like sample type, geography, age, time, hypervariable regions and sequencing method to set up a unified pulmonary baseline. We conclude that Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria are the predominant phyla in healthy people. At the genus level, the most common bacterial genera are Veillonella, Streptococcus, Prevotella, Neisseria and Fusobacterium. A significant difference exists at the bacterial genus level between the lung of healthy subjects and the normal tissues of patients, and geography impacts on the healthy baseline significantly. In addition, age, time, hypervariable regions and sequencing method all affect the baseline to various degrees. In healthy people, Ascomycota and Basidiomycota dominate the pulmonary fungal phyla, while bacteriophages are the predominated order in virome. Our investigation provides a healthy lung baseline for the study of lung microbiota, which is conducive to better finding lung disease-related pathogens.

Keywords: Microbiota, Lung, Next-generation sequencing, Healthy baseline.

1. Introduction

Microbiome include bacteria, archaea, fungi, and viruses as well as its DNA, RNA and proteins obtained by various means [1]. With the advance of next-generation sequencing [2] over the past decade, omics have continued to develop [3-6], expanding the study of the human microbiome from the gut [7] and other microbial-rich environments which had been previously thought sterile, including the bladder [8] lung [9,10] and so on. Unlike traditional culture methods which can only identify organisms that grow in specific media and under particular incubation conditions [11,12], culture-independent methods allow detection of all existing organisms, whether dead or alive [13]. These methods may extract all DNA from the samples and use various processes to detect the specific microbial DNA present in this mixed DNA extract [14]. These experiments usually refer to 16S rRNA gene sequencing [15,16], shotgun metagenomic sequencing [17,18]. Inherently, microbiome is an endeavor of multiple omics that generates “big data” [14]. For example, the relationships between lung microbes and lung related diseases such as lung cancer [19-21], asthma [22, 23], chronic obstructive pulmonary disease (COPD) [24, 25], and cystic fibrosis [26-28] have been highly discussed. Indeed, the occurrence, deterioration and treatment of lung diseases are all related to lung microorganisms in some ways. Recently, the baseline of pulmonary microbiota [29-31] is becoming more and more important, which would provide control grouping for identifying disease-related pathogens accurately [10]. However, given the invasive means required to obtain samples from sources such as bronchoscopy, BALF (bronchoalveolar lavage fluid), and lung tissue, the baseline of lung microbiota is often subject to ethical scrutiny capable of delaying progress. Here, we reviewed relevant studies about lung microbiota in PubMed database from 2017 to February 2022. Through our study, we hope to comprehensively summarize the baseline reference catalogue of lung microbes.
in healthy people based on the existing results, so as to provide reference and comparison for future studies.

2. Five phyla dominating in all studies about healthy subjects

In all the studies, the most frequent main phyla are Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria. Among them, Firmicutes were the primary phylum in all selected studies. In Fig.1, we found the main bacterial phyla in the United States [32-34], Asia [35-39] and Europe [40-43] remain completely consistent. In the normal tissues of patients [44-49], four main bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes) were shared with healthy subjects. By contrast, Fusobacteria was not the main bacterial phylum in the normal tissues of patients in all the studies, it's the biggest difference we found at the phylum level between patients and healthy people.

![Figure 1. Comparison of bacteria in Asian, North America and Europe at the phylum level. a. Comparison of top five pulmonary bacterial phyla of healthy people in Asian. b. Comparison of top five pulmonary bacterial phyla of healthy people in North America. c. Comparison of top five pulmonary bacterial phyla of healthy people in Europe. d. Comparison of major pulmonary bacteria at phylum level in three continents.](image)

3. Significant alternations in bacterial genera between healthy and diseased cohorts

In Fig.2a, we compared the top ten bacterial genera [44-49] with non-neoplastic normal tissue taken from patients with lung disease (usually lung cancer). Bacteria with high frequency belong to Pseudomonas, Corynebacterium, Propionibacterium, Sphingomonas and Acinetobacter. Pseudomonas aeruginosa, the most notorious of Pseudomonas, as an opportunistic pathogen and the main pathogens of nosocomial infection, can cause acute or chronic infection in patients [50,51]. In addition, Corynebacterium, which is considered as the part of the normal microbiota but also functions as a pathogen causing respiratory inflammatory diseases like tracheitis, pharyngitis,
rhinosinusitis, bronchitis [52-54]. While in table S1, high frequency bacterial genera in healthy people [32, 33, 35-37, 39-43] are Veillonella, Streptococcus, Prevotella, Neisseria, Fusobacterium, Haemophilus. In Fig.2b, we compared the bacterial composition of the patient's normal tissue to that of healthy people's lungs and found only one shared genus (Bacteroidetes). Therefore, it is doubtful that whether that lung microbiota obtained from patients’ normal lung tissue represents the baseline for healthy lungs.

**Figure 2.** Pulmonary bacteria among patients and comparison with the healthy at the genus level. a. Comparison of top ten pulmonary bacterial genera in patient's normal tissue. b. Comparison of major bacterial genera between healthy people and patients.

**Table S1.** The frequency of major genera in healthy subjects among included studies.

<table>
<thead>
<tr>
<th>genus</th>
<th>frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veillonella</td>
<td>10/10</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>9/10</td>
</tr>
<tr>
<td>Prevotella</td>
<td>8/10</td>
</tr>
<tr>
<td>Neisseria</td>
<td>5/10</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>5/10</td>
</tr>
<tr>
<td>Haemophilus</td>
<td>4/10</td>
</tr>
<tr>
<td>Granulicatella</td>
<td>3/10</td>
</tr>
<tr>
<td>Rothia</td>
<td>3/10</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>3/10</td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>2/10</td>
</tr>
<tr>
<td>Selenomonas</td>
<td>2/10</td>
</tr>
<tr>
<td>Actinomyces</td>
<td>2/10</td>
</tr>
<tr>
<td>Actinobacillus</td>
<td>2/10</td>
</tr>
<tr>
<td>Gemella</td>
<td>2/10</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>2/10</td>
</tr>
<tr>
<td>Enhydrobacter</td>
<td>2/10</td>
</tr>
<tr>
<td>Hydrotalea</td>
<td>2/10</td>
</tr>
<tr>
<td>Lachnospiraceae_incertae_sedis</td>
<td>2/10</td>
</tr>
<tr>
<td>Blautia</td>
<td>2/10</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>2/10</td>
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</table>

4. **The biogeography of healthy people's lung microbiota at the genus level**

To explore the effect of geography on the bacteria found in healthy people's lungs, we compared the bacterial composition of healthy people's lungs across different continents. Firstly, we compared the main bacterial genera of healthy people from different studies on the same continent to derive the continent’s main bacterial genera. In Fig.3a, we compared the studies from Asia [35-37,39] and found
that the bacterial genera with the highest frequency are Veillonella, Streptococcus, Staphylococcus. In Fig.3b, we found that the major European bacterial genera [40-43] are Streptococcus, Prevotella, Veillonella, Neisseria, Granulicatella. In Fig.3c [32, 33], Veillonella, Streptococcus, Prevotella, Fusobacterium are the same main bacterial genera in North America. Subsequently, in Fig.3d, we compared these continents about the main bacterial genera, Veillonella, Streptococcus and Prevotella. Furthermore, we found that Lachnospiraceae_incertae_sedis, Bacteroides, Hydrotalea, Staphylococcus, Enhydrobacter, Faecalibacterium, Blautia only appear in Asia, while Gemella, Granulicatella, Actinomyces are only found in Europe, which are absent from the composition of major pulmonary bacterial genera of healthy people on other continents (Asia and North America). The studies by Liu HX et al. [35] and Lou Y et al.’s [39] Shanghai-based studies, all had exactly the same top ten bacterial genera in the lungs despite all the differences in age and sampling methods. They were Prevotella, Bacteroides, Streptococcus, Faecalibacterium, Enhydrobacter, Staphylococcus, Veillonella, Blautia, Lachnospiraceae, Hydrotalea. However, Wang K et al.’s study in China [37] and Invernizzi R et al.’s study [41] in the UK showed that due to their geographical difference, although across similar age levels, similar sample types, and the same amplified hypervariable regions during sequencing, they only had Veillonella, Fusobacterium, Haemophilus in common as the major genera. In Fig.4a, we compared the composition of bacterial genera of healthy subjects from three studies across three different cities in China (Beijing, Shanghai, and Guangxi) and found that there were still large differences at the genus level (only one genus, Veillonella, in common) even when the subjects were in one country. In Fig.4b, in order to increase comparability, we did not limit the scope of comparison to the top ten major genera with the highest abundance but compared as many bacterial genera as possible. We found 11 identical bacterial genera in these three cities: Brevundimonas, Selenomonas, Treponema, Aggregatibacter, Haemophilus, Enhydrobacter, Fusobacterium, Veillonella, Porphyromonas, Capnocytophaga, Lactobacillus.

**Figure 3.** Comparison of bacteria in Asian, North America and Europe at the genus level. a. Comparison of top ten pulmonary bacterial genera in Asian healthy people. b. Comparison of top ten pulmonary bacterial genera in Europe healthy people. c. Comparison of top ten pulmonary bacterial genera in America healthy people. d. Comparison of major pulmonary bacterial genera in three continents.
5. Major pulmonary bacteria of healthy people in different ages at genus level

To compare the lung microbiota in healthy individuals of different age, we divided age into four groups (<16 years old as the child, 18-40 years old as the youth, 41-60 years old as the middle-aged, >60 years old as the elderly). Firstly, we compared the dominant bacteria in healthy subjects of the same age group. In Fig.5a, we found that Streptococcus, Prevotella, Veillonella are common to healthy and young populations [32, 39, 40] across three different continents (Asia, Europe and North America). At the same time, while Neisseria is common to youth in the North America [32] and Europe [40], there are more than 20 gram-negative bacteria in the genus Neisseria, which can colonize mucosal surfaces and are often found in a healthy upper respiratory tract. In addition to some non-pathogenic respiratory symbiotic species, such as Neisseria lactate [55], it also contains two important pathogens, N. meningitidis and N. gonorrhoeae which can cause respiratory infection [56]. Ralstonia and Prophyromonas occurred only once as the predominant bacterial genera in healthy people [40]. These are probably the main genera endemic to youth in Europe. In Fig.5b, Veillonella is the main bacterial genus common to all middle-aged healthy people [33, 35, 37, 41]. Other bacteria with high frequency occurrence are Streptococcus, Prevotella, Fusobacterium, Haemophilus. Among Haemophilus, Nontypeable Haemophilus influenzae (NTHi) epidemiology is a symbiotic pathogen that colonizes the airways of patients with respiratory disease, including COPD [57, 58]. The healthy subjects in the two studies [35, 37] originated from the same country and age group, but only one major genus (Veillonella) was identical, which may be due to their coming from different cities, variable regions of amplification during sequencing and different sampling methods. Pasteurellaceae appeared only once [33], and may be the main genus endemic to the lungs of middle-aged Americans. In Fig.5c, Streptococcus and Veillonella are the same main bacterial genera in healthy elderly people across geographies [36, 43]. Atopobium is unique to elderly North Americans, and Aggregatibacter, Treponema, Capnocytophaga, Dialister are unique to elderly Asians. In Fig.5d, we found that Veillonella and Streptococcus were the same main bacterial genera in the lungs of healthy people across all ages.
Figure 5. Comparison of bacteria in different age at the genus level. a. Comparison of top ten pulmonary bacterial genera in youth. b. Comparison of top ten pulmonary bacterial genera in the middle-aged. c. Comparison of top ten pulmonary bacterial genera in the elderly. d. Comparison of top ten pulmonary bacterial genera four age levels.

6. Other factors on defining the pulmonary bacteria at genus level in healthy cohorts

Nielsen R et al. [43], the first report on airway microbiome stability from a repeated bronchoscopy study, found that the lower airway microbiome changed after repeated bronchoscopy analysis over time. Across seven healthy controls, they completed three bronchoscopies at different times. In one of them, the Firmicutes and Proteobacteria had almost disappeared on the third examination but were present on the first and second examination. The changes in the species of Bacteroidetes and Fusobacteria in another healthy subject were also obvious. In the third round, the five phyla of the first bronchoscopy remained present, while only Proteobacteria (>75%) and Firmicutes (<25%) were found in the second round. The remaining healthy subjects also showed small fluctuations. Lung specimens obtained by bronchoscopy are susceptible to contamination from two main sources, bacterial DNA in the upper respiratory tract (via bronchoscopy through the pharynx [29]) and laboratory reagents [59]. Dickson RP et.al. [33] attempted to determine whether bronchoscopic sampling accurately reflected healthy lung microbiota by performing seven consecutive protective specimen scrubbing (PSB) and bilateral bronchialveolar lavage (BALs) on each subject. They found that BCC samples (Bronchoscopic contamination control specimens) had the lowest levels of mouth-lung community similarity (Bray-Curtis similarity), bacterial DNA (log10 number of 16S copies per reaction determined by real-time qPCR), and community richness (number of OTUs per 2,000 sequences). Furthermore, the sequences detected in the program and reagent control samples differed significantly from those detected in the PSB and BAL samples (P≤0.05 for all comparisons). The top three taxa (accounted for 29%±11%) of unused control protected specimen brushes were
Ruminococcus sp., Pseudomonas sp., and Acinetobacter sp. However, these three taxonomic groups were difficult to found (only accounted for 2%±1%) in PSB of healthy subjects. The common healthy person taxa in PSB and BAL of healthy people were Prevotella, Veillonella, and Streptococcus. Dickson RP et al. [33] confirmed that bronchial sampling and next-generation sequencing can reliably identify the members of the LRT (lower respiratory tract) bacterial community. In pediatric studies, the inclusion of lower respiratory tract samples from healthy controls is usually difficult to obtain because of the invasive sampling methods required. Cuthbertson L et al. [42] found no significant difference in bacterial diversity and community composition between paired blind brush and non-blind brush (R2 = 0.01, P = 0.36), indicating that the former is an effective method to collect airway microbiota. Therefore, after applying blind brush sampling methods to children, high levels of Streptococcus and Haemophilus were found.

The selection of different highly variable sequencing regions may also affect the results [60] leading to differences in research outcomes. We compared the results of different amplification regions and the techniques of 16S rRNA from healthy people and found that Streptococcus and Veillonella were the same main genera in different hypervariable regions (V1-V2, V3, V3-V4, V4) at the genus level (Figure 6). Indeed, metagenomic techniques show significant differences compared to 16S rRNA, probably because metagenomic sequencing is deeper, more high-resolution, and more sensitive to genera identification [61, 62]. As a result, shotgun more probably detect the microbiota at the genus level, such as Mycoplasma, Propionibacterium, Brucella, Bordetella, Xanthomonas, Burkholderia [36]. Interestingly, Mycoplasma, a special microbe without cell walls, exists in higher abundance in healthy people than other bacteria. Mycoplasma pneumoniae, the pathogen of human Mycoplasma pneumonia [63], remains the most abundant species.

![Figure 6](image_url)

**Figure 6.** Comparison of bacteria in different methods at the genus level. a. Comparison of top ten pulmonary bacterial genera of V3-V4 hypervariable regions. b. Comparison of top ten pulmonary bacterial genera of V4 hypervariable regions. c. Comparison of different amplification areas of 16S rRNA and technology at the major genus level.
7. **Common fungus in the lungs of healthy people**

At the fungal phylum level, Ascomycota and Basidiomycota were the most common fungi across three studies [36, 64, 65]. In Asia, at the fungal genus level, Tong X et al. [36] depending on the metagenomics technology found that Lindgomycyes, Dothidea, Westerdykella, Lophiotrema, Gloniopsis are the most abundant content of five kinds of fungi genera. In Europe, ITS1 sequencing was used in two studies to detect fungus in lung [64, 65], Fraczek MG et al. [64] found that young healthy individuals in Britain had a lower fungal burden, a higher proportion of Basidiomycete DNA, and a large proportion of Aspergillus spp complex. In addition, Candida spp and Cladosporium spp have been found in BALF of healthy human lungs. Martinsen EMH et al. [65] found that Candida (>40%), Malassezia and Sarocladium were the three highest concentrations of fungi at the genus level in elderly healthy controls in Norway.

8. **Bacteriophages governs the lung virome of healthy people**

Tong X. et al. [36] studied 23 non-smoking elderly people in Beijing and found 1420 viruses composed of 3683 virus strains using metagenomic sequencing in BALF. They found Caudovirales (a phage order) to be the most dominant and common order of viruses. Gamma retroviruses, which are commonly studied as vectors for gene therapy [66, 67], remain the most common virus genus in the lungs of healthy individuals, with Skunalikevirus, Lambdalikevirus, Sfi1nalikevirus, Sfi21dtinalikevirus also being common. Gregory AC et al. [68] also found that viruses infecting bacteria (bacteriophages) represented the vast majority of viruses in the lungs (>85% of the average viral community abundance) and that host genera span all 30 pulmonary viruses on the basis of phage abundance among young Americans. Across all viruses, phage Propionibacterium which was the most abundant among the 30 pulmonary viruses, accounts for 29% of the total viral community. The next most abundant phages were Streptococcus, Burkholderia, Escherichia, and Bacillus phages, each making up >10% of the mean viral community.

9. **Conclusion**

In summary, we inferred that Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria are the main bacterial phyla in healthy people. Furthermore, the most common bacterial genera are Veillonella, Streptococcus, Prevotella, Neisseria and Fusobacterium. The normal tissues found in healthy people and patients show significant differences at the bacterial genus level and geography has a significant impact on the health baseline. In addition, different age level, time, selection of hypervariable regions, and sequencing methods all affect the composition to various degrees. Ascomycota and Basidiomycota were predominant in the pulmonary fungal phyla of healthy people, while bacteriophages predominated in viruses. To a certain degree, our study makes up for the lack of a healthy baseline for lung microbiota, provides a healthy category for the study of lung related diseases and facilitates the discovery and exploration of pathogenic microbiota. Our study is the first comprehensive study concentrated on the composition of microbiota in the lungs of healthy people and its influencing factors. However, due to the small size of studies analyzed and the lack of comparability between various studies’ samples, our research may not be as robust as desired. Given the lack of specific microbial abundance in some studies, our study only involved the composition of the microbial community and only focused on the top five bacterial phyla and the top ten bacterial genera. Therefore, our conclusion may have some limitations. It is expected that relevant studies with large samples and meta-analysis of data may be carried out in the future to make up for the shortcomings of current studies.

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