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RESEARCH ARTICLE

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The effects of different lung parts, age, and batches on the lung microbiota of healthy rats

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ABSTRACT

Background: Rat models are valuable tools to study the lung microbiota in diseases. Yet the impacts of different lung parts, young and mature adult stages, and the different batches of the same conditions on the healthy rat lung microbiome have not been investigated.

Methods: The rat lung microbiome was analyzed to clarify the lung part-dependent and age-dependent differences and to evaluate the effects of several 'batch environmental factors' on normal rats, after eliminating potential contamination.

Results: The results showed that the contamination could be identified and excluded. The lung microbiome from left and right lung parts was very similar so one representative part could be used in the microbiome study. There were significantly different lung microbial communities between the young and mature adult groups, and also between the different feeding batches groups of the same repetitive feeding conditions, but a common lung microbiota characterized by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* as the most dominant phyla were present in all adult rats. It indicated that the experiment under the same condition of the same rats batch was needed to compare the difference in the lung microbiota and repeated experiments were necessary to confirm the results.

Conclusion: These data represented that the lung bacterial communities were dynamic and rapidly susceptible to environmental influence, clustered strongly by age or different feeding batches but similar in the different lung tissue parts. This study improved the basic understanding of the potential effects on the lung microbiome of healthy rats.

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Lung microbiome; healthy rat; 16S rRNA gene amplicon sequencing; core microbiome; respiratory microbiome

Introduction

With the development of techniques for high-throughput sequencing in microbiota research, the microbiota in the human body has been gradually characterized and found to play key roles in regulating disease states and maintaining normal homeostasis [1]. Early studies on the symbiotic human microbiome mostly focused on gut microbiota. Previously, it was believed that the lungs were sterile, as bacteria could rarely be cultured from healthy pulmonary tissue using conventional cultivation methods until new evidence has shown diverse and dynamic bacterial communities in lungs using a culture-independent method [2–4].

In the realm of various respiratory disorders [5], the composition of the lung microbiota undergoes significant alterations, exhibiting a strong correlation with changes in systemic and alveolar immunity [6, 7]. The inflammatory response of the host has the potential to

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profoundly impact the lung microbial community by altering the microenvironment, creating a conducive environment for the growth of specific microbial species. This, in turn, increases susceptibility to various lung diseases, including pathogen infections, asthma, and lung injuries. [6, 8, 9]. Increasing evidence suggests a significant correlation between variations in the lung microbiota and the physiological severity of pulmonary diseases, as well as their response to therapy. [2, 10, 11]. However, researches on healthy lungs are still rare though the lung microbiome has been recognized. The understanding of the primary functional traits of the lung microbiota and their impact on respiratory health remains limited.

Research on lung immunity and microbiology in humans is often constrained by ethical considerations, technical limitations, and small sample sizes. However, the utilization of animal models can offer valuable insights and novel information for investigating the lung microbiome. A key challenge in future lung microbiome research lies in the efficient utilization and development of animal models [12]. The rat (Rattus norvegicus) is widely employed as a prominent animal model in biological research, which has the advantages of rapid reproduction, mature and cheap feeding and management, and clear background for the known genomes. Compared to mice, rats are bigger, easier to dissect, and more resistant to various ailments, which is useful in the study of lung microbiota. It can be used to simulate almost every aspect of known human disease and has been widely used to test the efficacy and safety of drugs [13]. While some researchers have explored the age-dependent variations and effects of environmental changes on the lung bacterial community structure of healthy mice [14, 15], there is limited knowledge regarding the influence of external factors on the lung microbiome of healthy Sprague-Dawley rats. The majority of research studies have predominantly focused on investigating the lung microbiota during disease progression, leaving a gap in our understanding of healthy lung microbiota. Consequently, a more comprehensive exploration of the healthy lung microbiota is needed to fill this knowledge gap and enhance our understanding of its distinctive features distinct from disease-related changes.

In this study, the Sprague-Dawley rat from the same vendor and shipment and high-throughput sequencing technology was used to study the influence of environmental changes on the healthy rat lung microbiome. Previous studies on the lung microbiome in mice have focused on investigating the impact of lung lobes on microbial composition. It was found that samples collected from the upper and lower lobes of the right lung had similar microbial profiles, suggesting that samples from upper and lower lobes can be considered as a single lung compartment [16]. However, the influence of the different anatomical structures of the left and right lungs on the lung microbiome of healthy rats has remained unclear. Therefore, in this study, while investigating factors influencing the composition of the lung in healthy rats, samples were simultaneously collected from the left and right lungs to address this question. We raised rats from young adult age of 6 weeks to a mature adult age of more than 6 months under the same conditions. Whether the taxonomic composition across biological replicates was reproducible and distinct from negative controls was explored. The lung microbiome between young adult and mature adult rats, and from different feeding batches under the same feeding conditions were compared. We also investigated if there was a core lung microbiome that could be stable with time. The findings of this study have significant implications for the establishment of future experimental frameworks. For instance, these discoveries provide a foundation for subsequent investigations focusing on the evaluation of severe environmental alterations, such as antibiotic treatment. To better comprehend the factors influencing microbial community structures and functions, it is imperative to establish a comprehensive understanding of the undisturbed lung microbiota and characterize its age- or environment-dependent development. This knowledge will contribute to elucidating the underlying mechanisms that shape the lung microbiota and its response to various perturbations. Ultimately, it will enhance our understanding of the complex interactions within the lung microbiota and their potential implications.

Methods

Patient and public involvement

Patients or the public were not involved in this study.

Rats and ethics statement

The research followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals for animal experiments. It was approved by the Institutional Animal Care and Use Committee at the Guangzhou Medical University of China (Guangzhou, China; No. 2019-052 and 2019-159). The 6-week-old, male, Specific-Pathogen-Free (SPF) Sprague-Dawley (SD) rats weighing 200 ± 20 g were obtained from Guangdong Medical Laboratory Animal Center (Guangdong, China) SPF Biotechnology Co., Ltd. (Beijing, China).

Experimental design and sample collection

All rats were housed in a SPF room with alternating 12-hour light and dark cycles, maintained at the temperature of 24–26 °C and 50% humidity. They were provided with standardized food and water ad libitum. A one-week acclimation period was given to the rats in shared four-animal cages placed on free-standing shelves within the same cabinet, which shared a ventilation system [17]. To minimize potential cage effects, the rats were randomly assigned to different groups. To investigate the impact of different lung parts, ages, and batches on the lung microbiome, we determined the group sizes based on previous microbiome-related studies that utilized animal models [18–21]. Each group consisted of 4–6 rats with a consistent genetic background. This selection aimed to ensure sufficient sample size and control for potential confounding factors when analyzing the lung microbiome about the variables of interest. During this period, the feed litter and water source used in the same batch of rats were consistent. The model groups included the T_0 (Raised one week in August and the total raising time was 1 week, $n = 5$), $T_{6.1}$ (Raised from November to May and the total raising time was 24 weeks, $n = 4$), and $T_{6.2}$ (Raised from September to March and the total raising time was 24 weeks, $n = 6$) groups. Rats were euthanized under sodium pentobarbital anaesthesia after 1 week (young adult) or 24 weeks (mature adult) of feeding. Lung tissues were collected and immediately frozen in liquid nitrogen at −80 °C for future analysis. To minimize contamination, negative controls including PBS-rinsed surgical scissors ($n = 3$), PBS on the sampling bench ($n = 3$), and blank DNA extractions ($n = 3$) were used. These controls were employed to ensure the reliability of the whole lung tissue analysis. The right lower lobe and left upper part of the lung were collected to investigate the influence of different lung parts on the microbial composition.

Microbiota sequencing

Genomic DNA was extracted from lung lysate using the Qiagen genomic DNA isolation kit. The V3-V4 regions of the 16S rDNA genes [22] were amplified with specific primers and adapters. Three technical replicates were prepared for each sample and pooled together after purification for further sequencing. Sequencing was conducted on an Illumina NovaSeq 6000 platform using the 250 paired-end protocol at Biomarker Technologies Corporation (Beijing, China).

Data processing and statistical analysis

Raw sequencing data were demultiplexed according to barcodes and then quality-controlled using FastQC [23]. Forward and reverse end sequences of respective samples were merged using VSEARCH 2.21.1 [24]. To remove sequences that were filtered and chimeric sequences, the USEARCH 10 [25] software was utilized. Then the high-quality sequence analysis was performed by Quantitative Insights into Microbial Ecology (QIIME2, version 2-2022.2) pipeline [26]. We performed a comparative analysis by aligning the contaminant sequences with the sample sequences. Any single sequences that exhibited a coverage and similarity of 99% were considered as potentially contaminated and subsequently removed from the analysis. And the decontam R package were also used to check the results. Amplicon Sequence Variants (ASVs) were generated by UNOISE 3 [27] with high accuracy in quantifying abundance ratios in the case of a difference of only one base pair. Sequence reads that occur only once were discarded and ASVs present in negative control samples were removed before diversity analyses to avoid misleading conclusions [28]. To annotate taxonomic information for each representative sequence of the Amplicon Sequence Variants (ASVs), the SINTAX algorithm with the RDP training set v18 reference database was employed [25, 29, 30]. Random sub-sampling was performed to obtain equal sequencing depths among samples. The Chao1, Shannon, Simpson, and Goods' coverage index were measured by R software [31] with the vegan package [32]. The QIIME2 pipeline was employed to conduct Principal Coordinates Analysis (PCoA) and PERMANOVA tests. These analyses were conducted using Bray-Curtis distance metrics to assess differences in community structures. To investigate significant differences in taxon abundance among groups, the STMAP [33] software was utilized. For the *p-value* of STAMP analysis, Benjamini-Hochberg FDR correction is performed (*q-value*). Based on the assumption of normal distribution and homogeneity of variance, the Wilcoxon and Welch's *t*-test test were used for statistical analysis, with the significance set at $p < 0.05$ by the R package or STAMP. The PICRUSt2 algorithm [34] was used to estimate probable Kyoto Encyclopaedia of Genes and Genomes (KEGG) [35] function of the rat lung microbiome. The process sample reference code file can be found at: https://github.com/tingtinghuhuhu/16s-amplicon-analysis-procedure_1.

Results

Summary of sequence from all samples

A total of 1,909,237 16S rRNA gene reads were obtained with a mean ± SD of 127,282 ± 60,169 reads

across samples. The sequence data can be accessed at the ENA (European Nucleotide Archive) under the accession number PRJNA861950.

Comparison of the microbiomes from lung samples and negative controls

The difference in taxonomic composition between lung tissue and negative controls was first assessed. The negative control-derived samples yielded a total of 360,578 sequences that clustered into 2,036 ASVs. These mainly belonged to the phyla of Proteobacteria (42.51%), Firmicutes (20.14%), and Actinobacteria (17.18%), all of which were previously identified as reagent contaminants [28]. In comparison to the negative controls, the lung samples should contain a higher amount of bacterial DNA, leading to greater bacterial diversity. The lung tissue samples had greater community richness than negative control-derived samples ($p < 0.0001$ for all comparisons, Figure 1A) after being sub-sampled to the same sequences. There was a decrease in the average Bray-Curtis dissimilarity index from lung samples relative to that of negative controls (Figure 1B), as the low amount of bacterial DNA should be lower with an increase in sequencing stochasticity in the negative control-derived samples and the lung samples should have decreased sample-to-sample variation that represents greater replicability. The principal coordinates analysis (PCoA) was further used to assess the similarity of taxa identified in lung tissue and negative control specimens (Figure 1C). The lung samples showed prominently distinct from the negative controls ($p = 0.001$). The rank abundance analysis showed that the dominant taxa found in lung tissue had little resemblance to taxa from negative controls (Figure 1D). Together, these findings confirm that the presence of bacteria in lung specimens was not significantly influenced by procedural and sequencing contamination.

To enhance the objectivity and scientific rigor of our results, we performed additional sequence processing based on the sequences of negative controls. Sequences from lung samples that closely matched those in the negative controls (>99% similarity over at least 400 base pairs) were removed from the database to address potential contamination. This removal accounted for 43.9% of the sequences (1.8×10^4 out of 4.1×10^4 total reads), aligning with previous research findings of a 44% removal rate [16]. The bacterial taxa identified in lung tissue are reliable and can be used for further analysis. The read mapping process yielded a total of 1,312,707 reads that could be further grouped into 19,914 ASVs. Collectively, these sequences represented 26 phyla and 504 genera.

Comparison of the microbial composition between the left lung and right lung

As the left and right lung would usually need to be respectively used in the microbiome and pathological study at the same time, we cannot use the whole lung tissue in the lung microbiome research, and comparing the influence of the lung parts on the microbiome was essential. The rarefaction curves plateaued, which indicated this sampling depth was sufficient for subsequent group comparison (Figure S1A). After being sub-sampled to the same sequences according to the minimal sequences sample, the alpha and beta diversity analyses were performed to detect whether there was a difference in bacterial community composition within and between the left and right lung sample groups. Shannon, Simpson, Chao1, and ACE indexes in alpha diversity had no significant difference between the left and right lung parts (Figure 2A, Table S1).

The relative abundance of the lung taxa at the phylum and genera levels from the left and right lung sample groups was very similar to each other (Figure 2B–C). To assess the compositional similarities between samples, PCoA analysis was performed (Figure 2D–E). The results showed that samples from the left and right lungs clustered closely together, consistent with the observed phenotypes (Figure 2A–C). PERMANOVA permutation-based analysis of variance was conducted, confirming that the identified groups were not significantly different from each other ($p > 0.01$). Consequently, the microbial composition of both lung parts (left and right) was found to be similar, indicating that these samples could be treated as originating from a single lung unit.

Variability in the healthy lung microbiota communities with the age of the rat

To evaluate the developmental changes in the rat lung microbiome during aging, lung tissue samples were analyzed between the young adult rats (T_0) and mature adult rats ($T_{6.1}$) from the same vendor and shipment. As the $T_{6.2}$ group was from different batches, it was not compared with the T_0 group here. The rarefaction curves plateaued, which indicated this sampling depth was sufficient for subsequent group comparison (Figure S1B). A significantly increased ASVs richness ($p = 0.001$) in samples obtained when compared to $T_{6.1}$ and T_0 groups (Table S1). Shannon, Chao1, and ACE

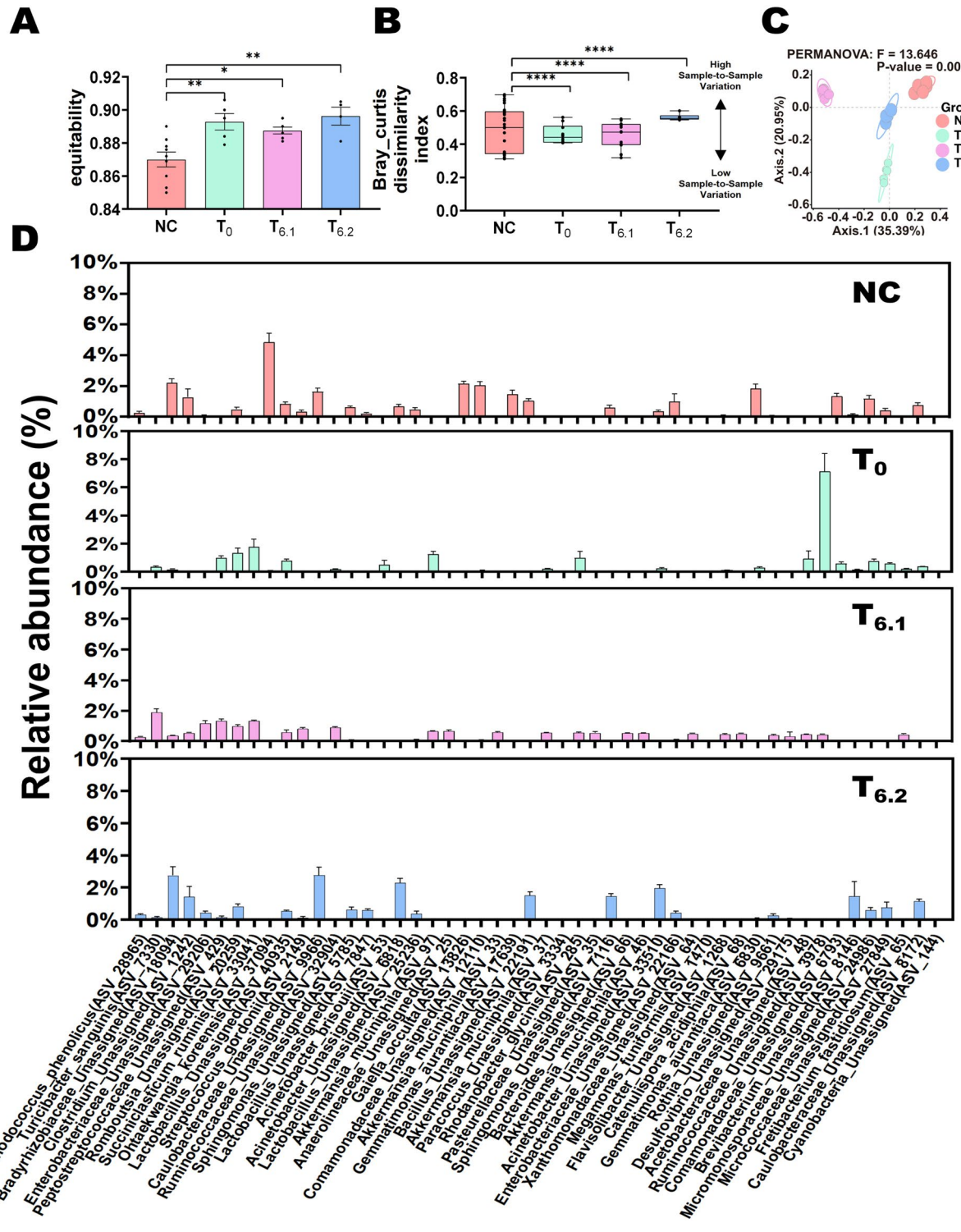

Figure 1. Alpha and beta diversity comparison between the lung samples and negative control samples. A, alpha diversity of bacterial communities in rat lung tissue and negative controls after normalizing to the same reads to account for variation in sequencing depth. Mean ± SEM and individual data points are shown. B, variation of bacterial communities in rat lung tissue among biological replicates compared to those in negative controls quantified by the Bray-Curtis dissimilarity index. Median, IQR, and all unique pairwise comparisons (individual data points) are shown. C, principal coordinates analysis (PCoA) for the lung tissue samples and negative controls. Individual data points represent specimens grouped by sample or control type. D, relative abundance of bacterial taxa in rat lung tissue and negative controls. Bars are ranked by mean abundance in lung tissue and represent mean ± SEM relative abundance of the top 50 bacterial taxa (ASVs) in whole lung tissue across sample types. Labels denote the most specific assigned taxonomic level and a unique identifier for each ASV. Pairwise significance was determined by (A, B) pairwise Wilcoxon test which was corrected using the Benjamini-Hochberg method and (C) PERMANOVA. Significance key: ns $p > 0.05$; *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; ****$p \le 0.0001$.

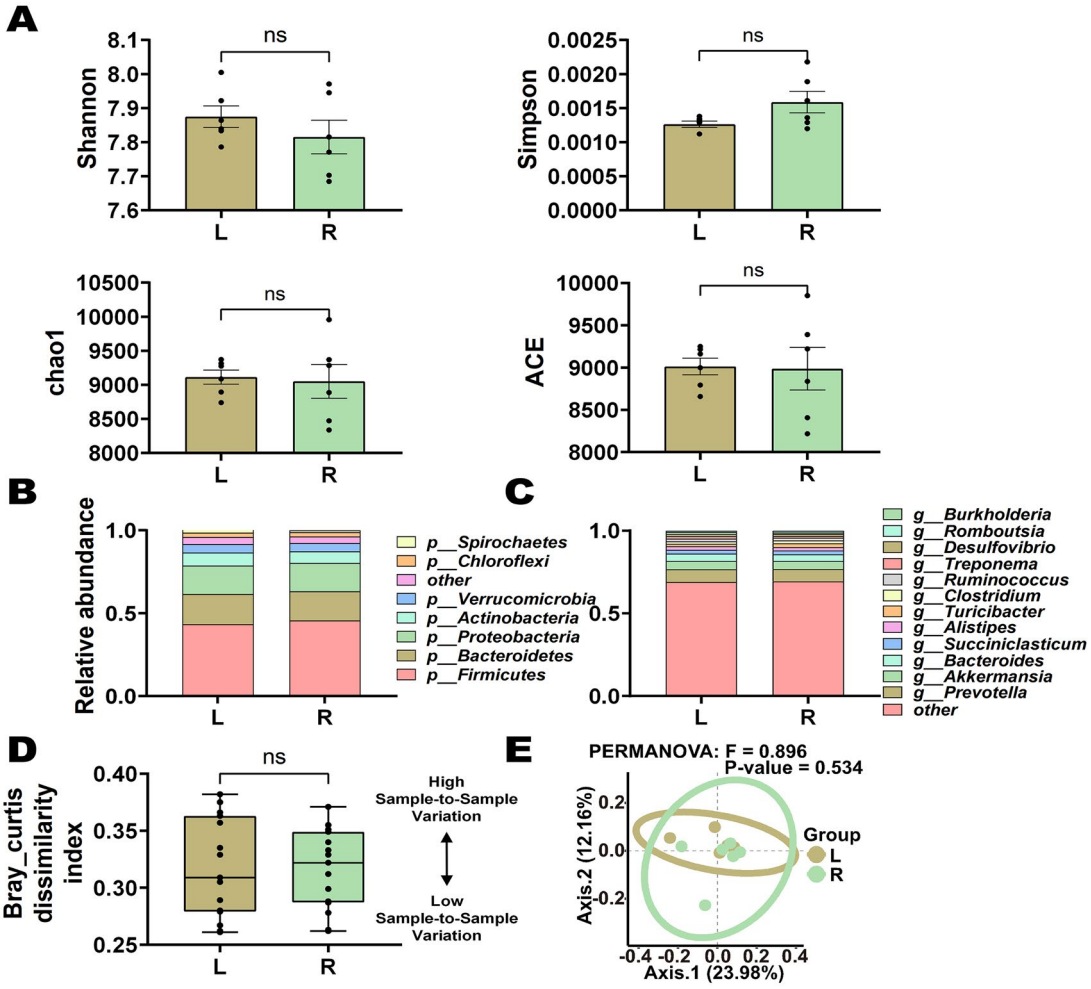

Figure 2. Lung microbiome composition in healthy rats of different lung parts groups. A, the alpha diversity (Shannon, Simpson, Chao1, and ACE indexes) between the left and right lung. The relative abundance of detected phyla (B) and > 1% genera (C) in the left and right lung groups. D, lung bacteria communities dissimilarity among rats in the same groups and from different lung parts groups. E, principal coordinates analysis (PCoA) for the microbiome composition of left and right lung tissue using Bray_curtis distances. Individual data points represent specimens grouped by sample or control type.

indexes in alpha diversity were significantly increased but Simpson indexes were significantly decreased for communities of rats belonging to the mature adult group ($T_{6.1}$) compared to the young adult group (T_0), all of which indicated the community diversity or richness were higher in $T_{6.1}$ than T_0 groups (Figure 3A and Table S1). Between-class PCoA based on Bray-Curtis distance was performed to investigate changes in lung microbial composition in rats of different ages (Figure 3B–C). Samples derived from the mature adult rats groups aged 30 weeks ($T_{6.1}$) clustered separately from those of rats aged 7 weeks (T_0), meaning that their bacterial communities were different from each other.

To identify the taxa that were significantly affected, we conducted a statistical analysis by comparing the relative abundance at both the phyla and genera levels. Figures 4A–D and S2A–D summarize the significantly differentially abundant taxa in response to age. Thus, the lung microbiota of the young adult rats group (T_0) was characterized mainly by the taxon *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes* at the phyla level and *Rhodococcus*, *Lactobacillus*, *Sphingomonas*, *Acinetobacter*, *Clostridium* at the genera level ($q<0.05$) (Figures 4C–D). The *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Chloroflexi* at the phyla level and *Prevotella, Akkermansia, Bacteroides, Succiniclasticum, Turicibacter, Ruminococcus*, and *Alistipes* at the genera level were significantly ($q<0.05$) associated with the mature adult group ($T_{6.1}$) (Figures 4C–D). However, the above differences between samples were mainly explained by both rat age and the raising environment. So we further compared the environmental effect on the lung microbiome.

Impact of environmental changes on the healthy lung community structure

After establishing that the lung communities of healthy rats cluster based on their age, we proceeded to investigate whether this variation in lung microbiota was

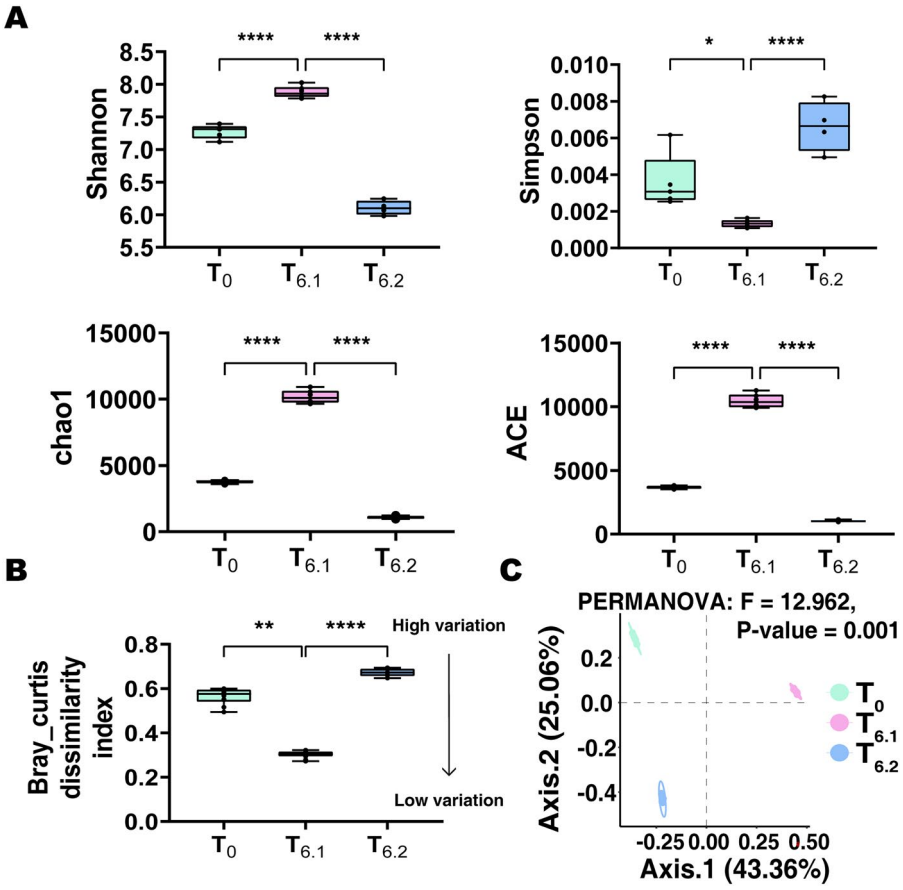

Figure 3. Species diversity between study groups. A, alpha diversity comparison as measured by Shannon, Simpson Chao1, and ACE index. Significant differences as calculated by Wilcoxon's test ($p < 0.05$). B and C, Beta diversity between lung microbiomes of the groups was measured by Bray_curtis Distance and visualized using PCoA. The significance of group dissimilarity as calculated by Wilcoxon's test and PERMANOVA was identified by the given *p-value*. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

indicative of each rat's environment. The influence of the different feeding batches on the lung microbiota was studied using the same healthy, genetically identical Sprague-Dawley rats from the same vendor, shipment manner, and in the same raising conditions with the controlled same temperature, humidity, light, and SPF environment. The influence of feeding batches under the above conditions was defined as the impact of batch environmental changes. We characterized the lung bacterial communities of $T_{6.1}$, and $T_{6.2}$, which raised rats from juvenile age of 6 weeks to mature adult age of 30 weeks twice. The groups exhibited a significant difference in alpha diversity measure ($p < 0.05$, Figure 3A). As depicted in Figure 3B–C, lung communities showed significant clustering by raising batches ($p < 0.01$ for all comparisons; PERMANOVA). However, the rat-to-rat taxonomic composition variation in the same raising batch was very little and showed similar communities. There were consistent significant functional differences by predicted metagenomes using PICRUSt2 between the different raising batch groups (Figure S3).

Our findings revealed considerable variation in the bacterial taxa identified in the lungs of rats from different raising batches (Figures 4A–B, 4E–F, and S2E–H). At the phyla level, Bacteria affiliated with *Firmicutes* (the mean relative abundance of the phylum *Firmicutes* was 39.94%), *Proteobacteria* (25.96%), *Bacteroidetes* (13.57%), *Actinobacteria* (11.35%), *Verrucomicrobia* (3.22%), *Gemmatimonadetes* (1.83%) and *Choroflexi* (1.05%) were dominating and significantly different between the different batch groups ($T_{6.1}$, and $T_{6.2}$). The genera *Prevotella* (5.05%), *Akkermansia* (3.05%), *Bacteroides* (2.77%), *Acinetobacter* (2.19%), *Clostridium* (2.14%), *Lactobacillus* (2.03%), *Sphingomonas* (1.65%), *Succiniclasticum* (1.38%), *Alistipes* (1.31%), *Turicibacter* (1.22%), *Ruminococcus* (1.06%), *Gemmatimonas* (0.97%), *Desulfovibrio* (0.74%) was distinctive. Besides, there were 12 taxa at the classes level, 19 taxa at the orders level, 15 taxa at the families level, and 4 taxa at the species that were significantly different between the $T_{6.1}$, and $T_{6.2}$ groups (Figure S2E–H).

Identification of the lung core microbial population of rat

We then sought to determine whether there was a 'core microbiome' that was consistently present in the

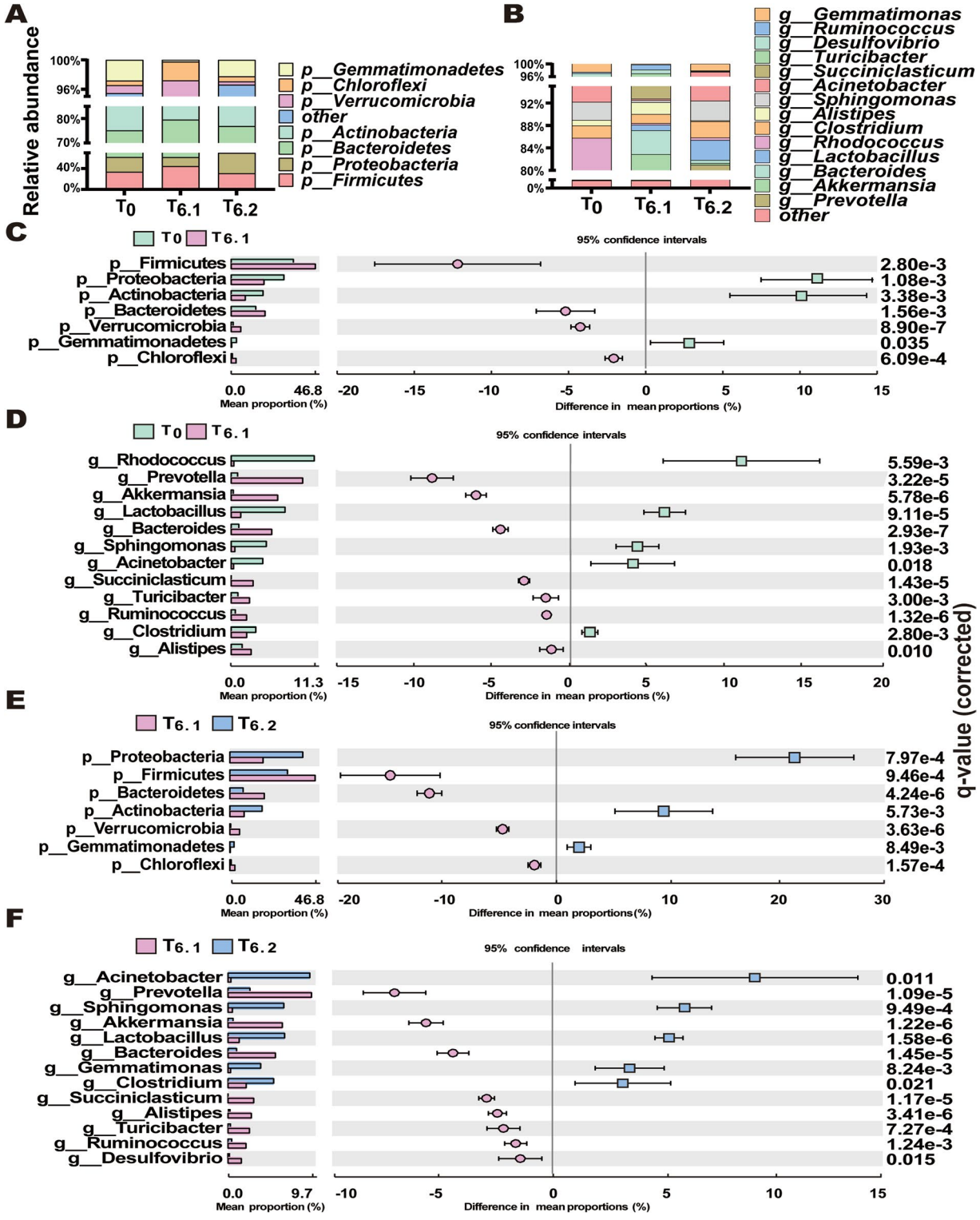

Figure 4. Lung microbiome composition in healthy rats across study groups. The relative abundance of detected phyla (A) and genera (B) in groups. Comparison of phyla (C) and genera (D) between the young adult (T_0) and mature adult ($T_{6.1}$) groups. Comparison of phyla (E) and genera (F) of different feeding batches ($T_{6.1}$ and $T_{6.2}$) groups. Significance was determined by Welch's *t*-test test and the *p-value* was corrected using the Bonferroni method (*q*-value). $^*p < 0.05$, $^{**}p < 0.01$.

lungs of all rats by comparing the adult rats to exclude the effect of age ($T_{6.1}$ and $T_{6.2}$). As shown in Figure 4A, The dominant bacterial phyla in the pulmonary microbial communities were *Firmicutes* (39.43% ± 6.83%), *Proteobacteria* (25.17% ± 10.62%), *Bacteroidetes* (13.97% ± 5.22%), *Actinobacteria* (10.99% ± 4.93%) and *Verrucomicrobia* (3.39% ± 2.26%). *Prevotella* (4.95%), *Clostridium* (2.14%), and *Lactobacillus* (2.03%)

represented the top three genera (Figure 4B). A total of 88/13978 ASVs with a relative abundance of 0.63% was shared across all lung samples of adult rats in the same batches, irrespective of age. When considering all the lung samples of T_0, $T_{6.1}$, and $T_{6.2}$ groups (Figure 4A–B), the dominant phyla of the lung microbiome were also *Firmicute*, *Proteobacteria*, *Bacteroidete*, *Actinobacteria*, and *Verrucomicrobia*. *Clostridium* and *Lactobacillus* at the genera level were also predominately detected in all samples. The lung communities of all experimental rats contained 49/19,913 common ASVs with a percentage of 0.03%.

Discussion

The microbiome of lung tissue collected from male healthy Sprague-Dawley rats of the same supplier that were kept under the same conditions was analyzed. The possible gender effects were excluded by focusing on male rats. To assess the impact of minor environmental change factors on the lung microbiome, the rats were repeatedly fed two bathes from 6 weeks to more than 30 weeks under almost identical controlling conditions with the same food, light, temperature, humidity, and collecting the lung tissue at the same season of different batches. Thus, we were able to explore the influences of different lung parts, the different ages, and the surrounding environment on the lung microbiota.

Given that the lungs have low bacterial biomass, it is important to consider the potential for microbial DNA contamination, which may overshadow the signal produced by the bacteria naturally present in the lungs [16, 36]. A substantial limitation in many studies focusing on pulmonary DNA is the absence of sequence data from relevant controls, which hampers the accurate interpretation of their results. In the present study, Lung tissue samples were dissected under sterile conditions. The sampling and assay negative controls were set up to identify potential contamination sources. The bacterial signal exhibited a notable disparity between the lung samples and the negative control samples (Figure 1). At the same time, our sequences and bacterial community were very consistent between rats in the same group. This observation suggested that the level of contamination, if present, was either minimal or evenly distributed among the rats, corroborating findings reported in a previous microbiome study conducted on mice [37]. The potential contamination sequences were eliminated from the data set to facilitate a comparison between the different lung sample groups of healthy rats. We also performed α and β diversity analyses on the dataset both before and after eliminating contaminants. The findings revealed that the overall trends remained consistent, as demonstrated in Figure 3 and Supplementary Material Figure S4. This suggested that the potential contamination observed in this study did not undermine the primary conclusions drawn from our research. Our results also indicated that the microbial composition of the left and right lung was similar which was consistent with previous reports that the bacterial community from the superior lobe and inferior lobe of the right lung was similar (Figure 2) [16].

Previous studies have found that the human and mouse lung microbiomes differed from each other in composition at the phylum level [15]. The phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* have been reported to dominate the lung microbiome at all the age stages of mice from neonatal to adult development [14, 15]. And *Proteobacteria* and *Firmicutes* were the most dominant phyla at all ages of healthy mice [15], while *Bacteroidetes* and *Firmicutes* were the most dominant phyla in healthy human lungs [38]. The observed difference in bacterial signal could potentially be attributed to the fact that mice are obligate nasal breathers, whereas human subjects utilize both their nose and mouth for breathing. In the healthy rats of our present study, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the most dominant phyla (Figure 4A), which was consistent with the previous study that found that the *Proteobacteria* was the dominant phylum of rat lung [39]. And the lung microbiome of the rat shared similar dominant phyla to mice and human lungs. *Achromobacter*, *Lactobacillus*, *Streptococcus*, *Actinobacillus*, *Bacillus*, and *Veilonella* were reported as the predominated genera of 8-week-old healthy mice [15]. And *Streptococcus*, *Prevotella*, and *Veilonella* were the dominant genera in the human lung microbiome [11, 40]. However, in this study, the rat lung microbiome is dominated by *Clostridium* (2.14%), and *Lactobacillus* (2.03%). *Streptococcus* which was reported as dominant genera in mice was also detected but only presented 0.73% as non-dominant genera in rat lungs of our result. These data appeared to show that the lung microbiome of the animal mode including mice and rats shared partial similarity to the human lung microbiome while harbouring many other taxa. This observation suggests that rats can be used as a model for studying the lung microbiome, but should also be compared with the available data from human clinical studies to get results that were suitable for humans.

The lung microbiota communities were significantly different between the young and mature adult rats (Figures 3–4 and S2). It was consistent with the age-dependent differences in the lung microbiota of mice [15] and other organs like gut [41]. Previous studies have reported that the microbial diversity in mice shows a progressive increase as they transition from neonates to adults, reaching its peak during the age range of 5–6 weeks and remaining relatively stable throughout their adult life up to 6–8 weeks of age [15]. Another study observed that the microbial communities in mice aged 4.5 and 9 months showed less dissimilarity. The oldest age group of 9 months exhibited an increase in diversity and similarity of lung communities, while higher variations were observed among samples of younger mice (5 days to 2 months) within the same age group [14, 42]. Overall, comparing the rat's lung microbial communities of T_0 and $T_{6.1}$, Our findings indicate a temporal development in the microbiome of mice, characterized by an increase in diversity and a more complex microbial community. This observation aligns with previous research conducted on mice. Besides the influence of age, our results indicated that under healthy conditions, the lung microbiota of rats is highly diverse and influenced by minor environmental changes from the comparison of the $T_{6.1}$ and $T_{6.2}$ groups (Figures 4 and S2). It was supported by the results that the lung microbiota of healthy mice was highly variable depending on the cage, and transportation of mice, and associated with the natural immune status of the host lung [43]. Complex interactions occur between microorganisms within the microbiota and between microorganisms and their environment, influencing the composition and function of the microbiota [14]. As the water had been reported to be the greatest contribution to the oral microbiome and the oral microflora was correlated to the lung microbiome in rats [44], the food including the food and the feeding patterns were kept similar to study different feeding batches with surrounding minor environmental changes in our study. The air bacteria, the main source of the lung microbiota, disperse into the lung, and then the composition of the lung microbiota is maintained through bacterial reproduction, immigration, and elimination processes [45] to form the finally presented lung microbiome of the lung tissue. These factors contributed to the temporal dynamics of microbial communities of the healthy lung microbiome, which may explain the differences in feeding batches difference in our study. However, relative stabilization of lung microbiota was present at the adult age of rats with lung core common microbial population. Indeed, as we have discovered in another study [17], the lung microbiota can self-regulate and maintain homeostasis. By perturbing the lung microbiota with varying concentrations of ampicillin under controlled other consistent environmental factors, we observed significant differences in community structure only at high antibiotic concentrations. This highlights the delicate balance and self-stabilizing capacity of the lung microbiota.

In this study, including more age sampling time points would provide a more complete understanding of the development of the lung microbiome in rats. The more detailed subdivision of influencing factors, such as proximal vs. distal lung, may also need to be taken into consideration. The fungi and viruses were also important components of the total lung microbiota, especially the bacteriophages that have been reported to influence gut or lung microbiology and indirectly have adverse effects on health [46]. Further studies could analyze whether the above-mentioned factors could influence the lung microbiome.

Conclusion

To our knowledge, research describing the different ages of healthy rat lung microbiota and the impact of minor environmental changes on these communities is lacking. This work provided insights into the influence of different lung parts, young and mature adults, and the environmental changes on the rat lung microbiota. Overall, our results suggested that negative controls should be considered to exclude the potential contamination that might falsely increase the taxonomic overlap between the groups. The microbial composition of the left and right lungs was similar and one representative part would be enough for the study. The healthy lung microbiome was dynamic though in adulthood from 6 weeks to 30 weeks. Further, the microbiota of healthy rats clustered strongly by the environmental changes of different feeding batches at the almost same raising condition. Hence, the reduplicative animal mode assays of different batches were necessary and suggested to get a robust result. However, the lung core common microbial population was present in adult-age of rats with Firmicutes, Bacteroidetes, and Proteobacteria as the most dominant phyla. Such an understanding of undisturbed lung microbiota was essential for creating new interventions for curing lung diseases. This study can serve as a basis for future experimental frameworks to investigate the impact of severe environmental changes such as tobacco smoke or antibiotic treatment and to understand the possible cause and effect of the lung microbiota in the disease.

Author contributions

PXR, YMZ, BL, and GYL conceived the study, directed the project, and designed the experiments. HNJ obtained the samples. TTH performed the microbial sequencing analysis. PC interpreted the results, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

We declare no conflict of interest.

Consent for publication

Not applicable.

Disclosure statement

The authors report no conflict of interest.

Ethics statement

The care and use of the animals complied with regulations designated by the Chinese Association for Laboratory Animal Science Policy. All of the experimental protocols were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University (No. 2019-052 and 2019-159). Please contact the author for data requests.

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Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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