



Comparative analysis of the pulmonary microbiome in healthy and diseased pigs

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Received: 29 April 2020 / Accepted: 3 September 2020 / Published online: 17 September 2020
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Abstract

The lungs possess an effective antimicrobial system and a strong ability to eliminate microorganisms in healthy organisms, and were once considered sterile. With the development of culture-independent sequencing technology, the richness and diversity of porcine lung microbiota have been gaining attention. In order to study the relationship between lung microbiota and porcine respiratory disease complex (PRDC), the lung microbiota in healthy and diseased swine bronchoalveolar lavage fluids were analyzed and compared using the Illumina MiSeq sequencing platform. The predominant microbial communities of healthy and diseased swine were similar at the phylum level, mainly composed of Proteobacteria, Firmicutes, Tenericutes, and Bacteroidetes. However, the bacterial taxonomic communities of healthy and diseased swine differed at the genus level. The higher relative abundances of *Lactococcus*, *Enterococcus*, *Staphylococcus*, and *Lactobacillus* genera in healthy swine might provide more benefits for lung health, while the enhanced richness of *Streptococcus*, *Haemophilus*, *Pasteurella*, and *Bordetella* genera in diseased swine might be closely related to pathogen invasion and the occurrence of respiratory disease. In conclusion, the observed differences in the richness and diversity of lung microbiota can provide novel insights into their relationship with PRDC. Analyses of swine lung microbiota communities might produce an effective strategy for the control and prevention of respiratory tract infections.

Keywords Porcine respiratory disease complex · Bronchoalveolar lavage fluid · Lung microbiota · Symbiotic microbiota · Microbial community

Introduction

Previous studies of porcine commensal and symbiotic microbiota mainly focused on those inhabiting the intestinal tract; however, microbial communities colonizing the respiratory tract have been poorly investigated. The microbiome diversity and composition of the respiratory tract play an important role in regulating host immune homeostasis and preventing the porcine respiratory diseases complex (PRDC) (Niederwerder 2017). Increased diversity of the symbiotic microbiome has been shown to be associated with systemic

virus infection in primary lung pathology; therefore, the modulation of symbiotic microbiomes could be used to prevent pulmonary infections induced by porcine reproductive and respiratory syndrome (PRRS), porcine circovirus type 2 (PCV2) and other respiratory pathogens (Niederwerder et al. 2016; Wang et al. 2018). The development of a symbiotic microbiome in early life might be impacted by birth mode, antibiotic usage, diet composition, living environment, and contact with pathogens. A large amount of evidence has demonstrated that the relationship and balance between the symbiotic microbiome and host could influence immune regulation and respiratory disease infection (O'Dwyer et al. 2016; Wang et al. 2017).

The lungs were previously considered to be sterile, as it was difficult to obtain bronchoalveolar lavage samples that had not been contaminated with microbes from the oral and upper respiratory tracts. With the development of culture-independent next-generation sequencing technologies, the microbial communities that inhabit the lower respiratory

Communicated by Stefan Hohmann.

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tracts began to be collected and investigated. The taxonomic profiles of lung microbiota are remarkably similar to oral microbiota, indicating that microorganisms inhabiting the lung probably originated from the oral cavity through microaspiration (O'Dwyer et al. 2016). Though the lung has a large surface area and is continuously exposed to entering environmental microorganisms, an effective antimicrobial system can selectively eliminate entering microbiota and maintain a balanced microbial ecology of the pulmonary alveolar (Wypych et al. 2019). Metagenomic analysis of pig lung microbial communities revealed an abundance of bacteria, and the most common microbial families were identified as Mycoplasmataceae, Bradyrhizobiaceae, Flavobacteriaceae, and Pasteurellaceae (Siqueira et al. 2017). Studies on the relationship between swine lung lesion phenotype and lung microbiota diversity demonstrated that *Mycoplasma*, *Ureaplasma*, *Sphingobium*, *Haemophilus*, and *Phyllobacterium* were the main microbial communities in lungs with severe lesions, whereas *Methylobacterium*, *Prevotella*, *Sphingobium*, and *Lactobacillus* were most abundant in healthy lungs (Huang et al. 2019). Microbial ecology analyses performed by Illumina MiSeq sequencing revealed that the dominant bacteria were *Haemophilus parasuis*, and *Mycoplasma hyorhinis* in the lung of PRRSV-challenged pigs, which is consistent with the clinical observation that PRRSV-infected pigs are always co-infected with bacteria (Jiang et al. 2019).

PRDC is commonly caused by complex infection of viral or bacterial pathogens and can induce significant animal morbidity and mortality in the swine industry. Adverse environmental and management conditions could significantly impact pig microbiome diversity and immune development, influencing porcine growth performance and in turn cause serious economic losses. In respiratory disease states, the homeostasis of dynamic microbiota ecosystems and immune responses are altered, causing the dysregulated lung microbiome to drive the inflammatory response and promote the occurrence of respiratory infection (Dickson et al. 2015; Yang et al. 2019). Microbial compounds (such as lipopolysaccharide) produced by lung microbiota can be sensed by pattern recognition receptors (PRRs) causing naive T cells to change to Th1 or Th2 cells to maintain homeostasis of the pulmonary immune system (Shekhar et al. 2017). Toll-like receptors (especially TLR4) that are constitutively expressed in alveolar macrophages and bronchial epithelial cells play an important role in activating the innate and adaptive immune responses (Nembrini et al. 2011). In fact, the expressed levels of MHC Class II, interleukin-10 (IL-10), and transforming growth factor beta (TGF- β) in alveolar macrophages are much lower than macrophages from other organs (Wang et al. 2017). Evidence has indicated that lung microbiota could be influenced by intestinal microbiota; therefore, the host–microorganism interactions between the

mucosal tissues form a bi-directional communication of the gut–lung axis (Budden et al. 2017; Marsland et al. 2015). Therefore, swine lung could serve as an ideal model to research the role of lung microbiota in respiratory diseases.

In the present study, a total of 28 pulmonary lavage-fluid samples were collected and DNA was extracted. The V3–V4 region of the 16S rRNA gene was sequenced and the microbial communities were investigated and analyzed.

Materials and methods

Ethics statement

All animal work carried out in this study was in accordance with the guidelines and recommendations of the experimental animal administration and ethics committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (under permit number SHVRI-P-2019110802). The protocol was approved by the experimental animal administration and ethics committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Sample collection

A total of 28 Duroc \times Landrace \times Yorkshire crossbreed pigs were purchased from a commercial pig farm located in Shanghai city (30°51'53"N, 121°38'23"E). All the pigs were separated into two groups: healthy (named SH group, $n = 8$) and diseased (named SD group, $n = 20$). The pigs were diagnosed by veterinarians with over 5 years of clinical experience. Physical examination revealed pigs with cough, fever, joint swelling, wheezing, and other PRDC clinical symptoms that were diagnosed as diseased, while pigs that had never experienced PRDC or other diseases were diagnosed as healthy. After fasting overnight, the pigs were intravenously injected with 20 mg/kg xylazine hydrochloride for euthanasia at the age of 270 ± 3 days, and whole lungs were immediately removed. Lung lavage-fluid samples were collected by pouring sterile phosphate-buffered saline (PBS) buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate and 2 mM potassium phosphate) into lungs, after gently kneading for 2 min. Then the lavage fluids were centrifugated at 5000 rpm for 10 min (Huang et al. 2019; Jiang et al. 2019). All pellets were stored at -80 °C until DNA extraction.

DNA extraction and Illumina MiSeq sequencing

The genomic DNA of bronchoalveolar lavage fluids was extracted using QIAamp Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. For PCR

amplification, the V3-V4 region of the 16S rRNA gene was amplified using universal bacterial forward primer (338F, 5'-ACTCCTACGGGAGGCAGCAG-3') and reverse primer (806R, 5'-GGACTACHVGGGTWTCTAAT-3'). Each PCR reaction was performed in a 25- μ L reaction mix containing 2 \times Phanta Max Buffer, 10 mM dNTP Mix, 1 mM of each specific primer, 0.5 U of FastPfu Polymerase, and 10 ng template DNA. The PCR reactions were amplified using the following conditions: 1 cycle of initial denaturation at 95 °C for 3 min, 28 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s, followed by the last cycle of elongation at 72 °C for 10 min. The PCR products were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified by a QuantiFluor™-ST (Promega BioSciences LLC, Sunnyvale, CA, United States) according to the manufacturer's instructions. The purified amplicons were paired-end sequenced on an Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Bioinformatics analysis of sequencing data

The sequenced fastq files were demultiplexed, quality-filtered by Trimmomatic, and merged by FLASH v1.2.11. Operational taxonomic units (OTUs) were then clustered for 97% similarity using UCLUST cluster algorithm in USEARCH; however, chimeric sequences were discarded by UCHIME (Chen et al. 2017; Li et al. 2017). The 16S rRNA gene sequence was analyzed by RDP v2.2 classifier for taxonomy assignment. A Venn diagram was generated to show shared and unique OTUs between the two groups, and rarefaction curves were analyzed to confirm sequence coverage of all samples. For alpha diversity estimations, richness (ACE and Chao indices) and diversity (Shannon and Simpson indices) were conducted using mothur software. The relative abundances of lung bacterial communities were compared using STAMP software at the phyla and genera levels, respectively. Beta diversity analysis was performed using QIIME v1.8.0, and principal coordinate analysis (PCoA) analysis was conducted with weighted UniFrac distances. A heatmap was created with the R-package at the genus level. Significant differences in OTU abundance between the two groups were analyzed by the non-parametric Wilcoxon rank-sum test (Wang et al. 2018). The PICRUST package was used to predict the contribution of bacterial community genes for potential function through the EggNOG database.

Statistical analysis

Each experiment was independently completed in triplicate to derive an average and standard deviation. Data were presented as mean \pm SEM when indicated. A *T* test was used for

statistical analyses and differences were considered significant at $P < 0.05$.

Results

OTU analysis of the sequencing data

All samples were sequenced using 16S rRNA gene sequencing and comparisons were analyzed between the two groups. Primers and barcodes from raw sequencing data were paired to remove low-quality reads, and then tag sequences were generated from the merged clean sequences after OTU (97% sequence similarity) clustering. A total of 1,327,648 quality-filtered and chimera-checked sequences were obtained with an average length of 426.72 bp among the 28 samples (Table 1). After removing OTUs with average relative abundances lower than 0.01%, a total of 552 qualified OTUs were obtained for this study. Taxonomic analysis of these 552 qualified OTUs revealed 23 bacteria phyla, 45 classes, 90 orders, 154 families, 271 genera, and 395 species.

The number of unique and shared OTUs between the two groups was demonstrated by Venn diagrams (Fig. 1a). The total number of OTUs in the SD group was much higher than that in the SH group (431 versus 296), while 175 OTUs were shared between the two groups, indicating that the shared OTUs might be common inhabitants of lung microbiota. Rarefaction curves were measured using the mothur program, and Sobs estimators revealed that current sequence coverage was adequate for microbial community analysis (Fig. 1b).

Microbial diversity of lung microbiota

For alpha diversity analysis, bacterial community indices (Chao1 and Ace) were calculated for richness analysis, while Shannon and Simpson indices were calculated for diversity analysis. ACE and Chao1 indices indicated that the bacterial richness of the SH group was significantly lower than the SD group (Fig. 2), while Shannon and Simpson indices revealed that the bacterial community diversity of the SH group was also significantly lower than the SD group. Results of alpha diversity analysis revealed that the bacterial community richness and diversity of healthy pigs were both lower than the diseased pigs. For beta diversity analysis, principal coordinates analysis (PCoA) based on weighted UniFrac distance revealed that the two different experiment groups were segregated into different community clusters (Fig. 3).

Table 1 Summary of sequencing data identified in swine lungs

Amplified Region	Samples	Sequences	Bases (bp)	Average length
338F_806R	28	1327648	566543547	426.72

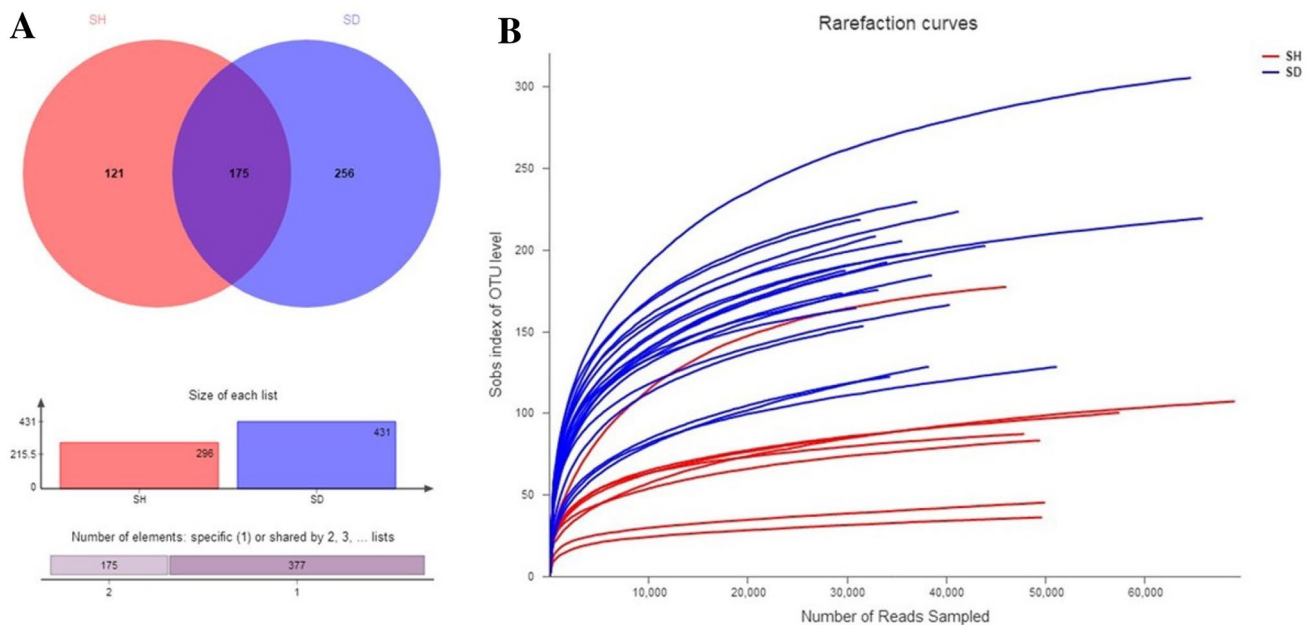


Fig. 1 Venn diagram displayed the shared and unique OTUs between the two groups and the rarefaction curves were generated by Sobs estimators. The total number of OTUs in the SD group was much higher than that in the SH group (431 OTUs versus 296 OTUs), while

175 OTUs were shared between the two groups. Rarefaction curves revealed that the current sequence coverage was adequate for microbial community analysis

Microbial composition of lung microbiota

To assign taxonomic composition of swine lung microbiota, the RDP classifier was used to compare the bacterial community structure at both the phylum and genus levels. A total of 23 different phyla and more than 271 genera were identified across all sequenced samples, and the most predominant microbiota are shown in Table 2.

As shown in Fig. 4a, the swine lung microbial community of the two groups at the phylum level were similar. The relative abundance of Proteobacteria and Firmicutes comprised the main microbial community in both the SH (87.67%) and SD (88.30%) groups. However, the microbial composition of Tenericutes in the SH group (9.94%) was much higher than in the SD group (3.19%), while the microbial composition of Bacteroidetes in the SH group (2.09%) was significantly lower than the SD group (7.79%).

The microbial communities of swine lung microbiota at the genus level were also analyzed. As shown in Fig. 4b, the microbial composition between the two groups at the genus level differed noticeably. In the SH group, the most predominant genera with relative abundances of over 1% were certified to be *Escherichia-Shigella* (42.51%), *Mycoplasma* (9.94%), *Lactococcus* (8.64%), *Macrococcus* (7.08%), *Enterococcus* (4.93%), *Acinetobacter* (4.28%), *Enterobacter* (4.08%), *Staphylococcus* (3.85%), *Streptococcus* (3.36%), *Aeromonas* (3.35%), *Citrobacter* (2.82%), and *Raoultella* (1.29%). While in the SD group, the most predominant

genera were *Aeromonas* (24.33%), *Psychrobacter* (8.20%), *Acinetobacter* (7.88%), *Vagococcus* (7.42%), *Streptococcus* (7.07%), *Macrococcus* (6.60%), *Mycoplasma* (3.19%), *Citrobacter* (2.48%), *Empedobacter* (2.48%), *Escherichia-Shigella* (1.89%), *Peptostreptococcus* (1.83%), *Morganella* (1.78%), *Soonwooa* (1.75%), *Haemophilus* (1.73%), *Shewanella* (1.61%), and *Enterococcus* (1.06%). Significant differences in lung microbiota between the two groups were observed, the relative abundances of *Lactococcus*, *Enterococcus*, and *Staphylococcus* were much higher in the healthy group, while *Streptococcus* and *Haemophilus* were much higher in the diseased group.

Comparison of lung microbial communities between the two groups

We used the Wilcoxon rank-sum test to compare the mean percentage of predominant genera between the SH and SD groups. The significant differences of the relative abundance of swine lung microbiota is shown in Fig. 5. The percentages of *Escherichia-Shigella* (42.51% versus 1.89%, $P < 0.001$), *Lactococcus* (8.64% versus 0.32%, $P < 0.001$), *Enterobacter* (4.08% versus 0.55%, $P < 0.01$), and *Staphylococcus* (3.85% versus 0.000017%, $P < 0.001$) were significantly higher in the SH group than the SD group. However, the percentages of *Aeromonas* (24.33% versus 3.35%, $P < 0.01$), *Psychrobacter* (8.20% versus 0.000029%, $P < 0.001$), *Acinetobacter* (7.88% versus 4.28%, $P < 0.01$), *Vagococcus* (7.42% versus

Fig. 2 Alpha diversity analysis of the lung microbial community. The richness estimators ACE (a) and Chao1 (b) and the diversity indices Shannon (c) and Simpson (d) were calculated. ACE and Chao1 indices indicated that the bacterial richness of SH group was significantly lower than the SD group, while Shannon and Simpson indices revealed that bacterial community diversity of SH group were also significantly lower than the SD group

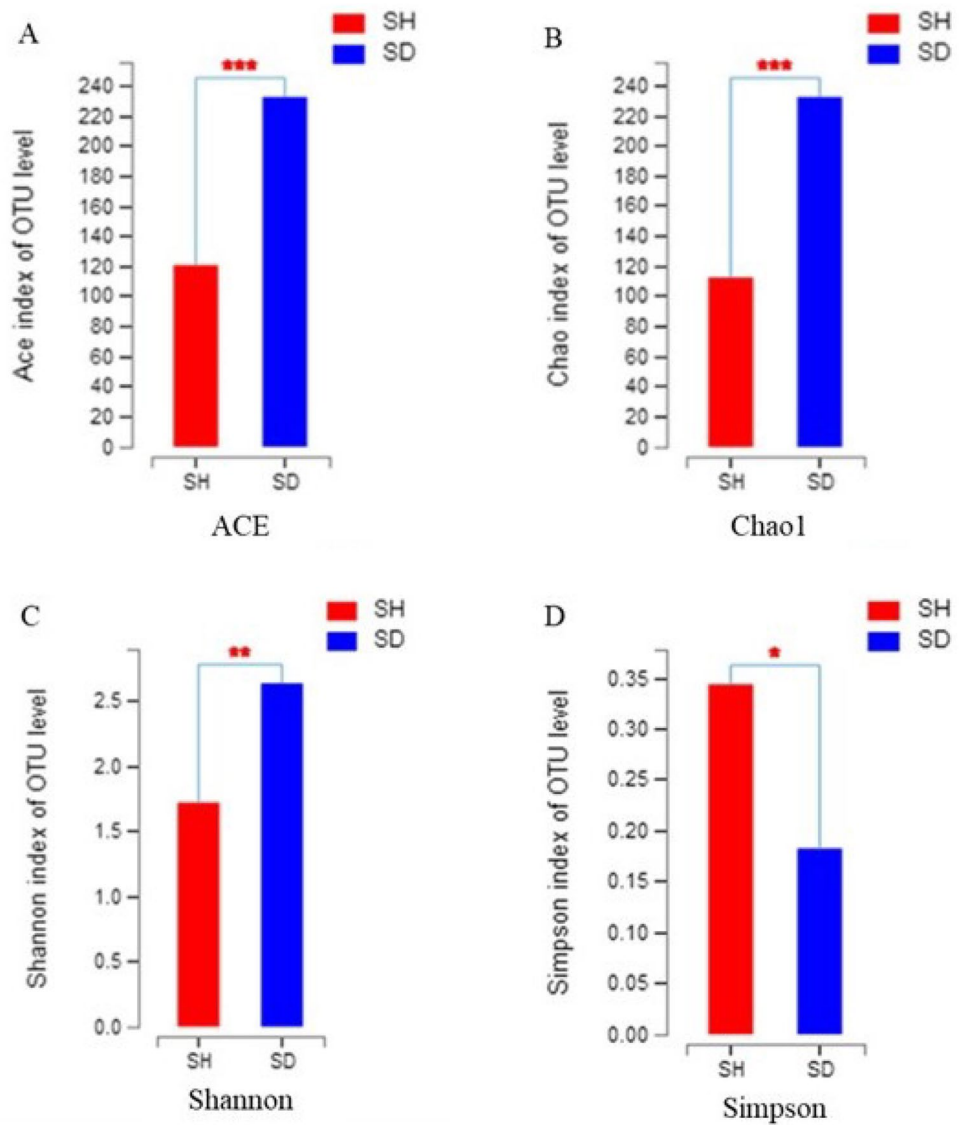


Fig. 3 Plot of principal coordinate analysis (PcoA) of the the lung microbial community. The two different experiment groups were segregated into different community clusters

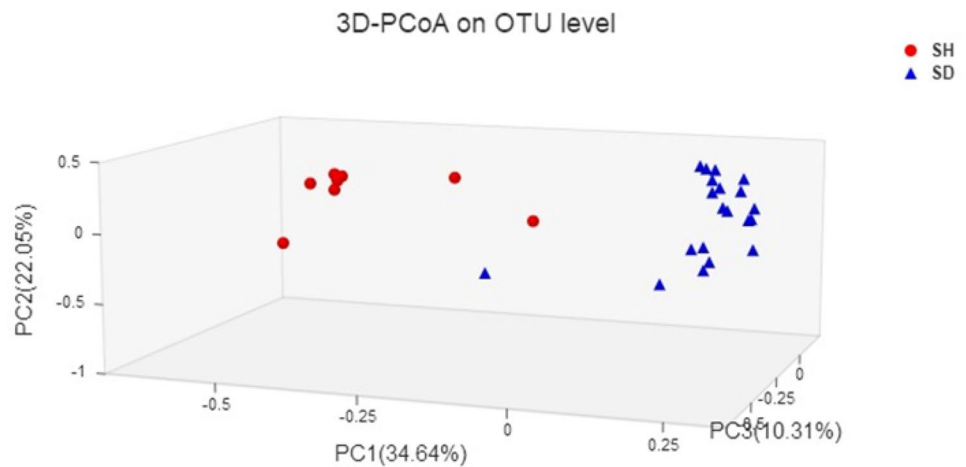


Table 2 The predominant microbiota in the healthy and diseased groups

Sample group	Taxonomic level	Phyla	Relative abundance (%)
SH group	Phylum	Proteobacteria	59.12
		Firmicutes	28.55
		Tenericutes	9.94
		Bacteroidetes	2.09
	Genus	<i>Escherichia-Shigella</i>	42.51
		<i>Mycoplasma</i>	9.94
		<i>Lactococcus</i>	8.64
SD group	Phylum	Proteobacteria	54.22
		Firmicutes	34.08
		Tenericutes	3.19
		Bacteroidetes	7.79
	Genus	<i>Aeromonas</i>	24.33
		<i>Psychrobacter</i>	8.20
		<i>Acinetobacter</i>	7.88
		<i>Vagococcus</i>	7.42
		<i>Streptococcus</i>	7.07

0.05%, $P < 0.001$), *Empedobacter* (2.48% versus 0.00005%, $P < 0.001$), *Peptostreptococcus* (1.83% versus 0.000007%, $P < 0.001$) were significantly higher in the SD group than the SH group. Therefore, the predominant genera compositions of the swine lung microbial community differed significantly between the SH and SD groups.

A heatmap displaying the top 50 genera, according to relative abundance, was generated and the color of the

spots in the panel indicate the abundance percentages of the OTUs in each sample (Fig. 6). Results indicated that relative abundances of *Haemophilus*, *Pasteurella*, and *Bordetella* were higher in the SD group than the SH group; however, the percentage of *Lactobacillus* was higher in the SH group than the SD group. The microbial community differences between the two groups indicated that lung microbiota might be associated with swine respiratory disease.

PICRUSt functional prediction

Predicted functions were calculated based on PICRUSt in the EggNOG database (evolutionary genealogy of genes: Non-supervised Orthologous Groups, <https://eggnoг.embl.de/>), and 23 pathways related to swine respiratory diseases were identified (Fig. 7). The abundance of microbial genes involved in RNA processing and modification, extracellular structures, nucleotide transport and metabolism, energy production and conversion, cell motility, and carbohydrate transport and metabolism were much higher in the SH group. However, microbial genes related to signal transduction mechanisms, lipid transport and metabolism, coenzyme transport and metabolism, defense mechanisms, amino acid transport and metabolism, chromatin structure and dynamics, and cytoskeleton were much higher in the SD group. The predicted functions of extracellular structures and cytoskeleton were significantly different between the two groups ($P < 0.05$).

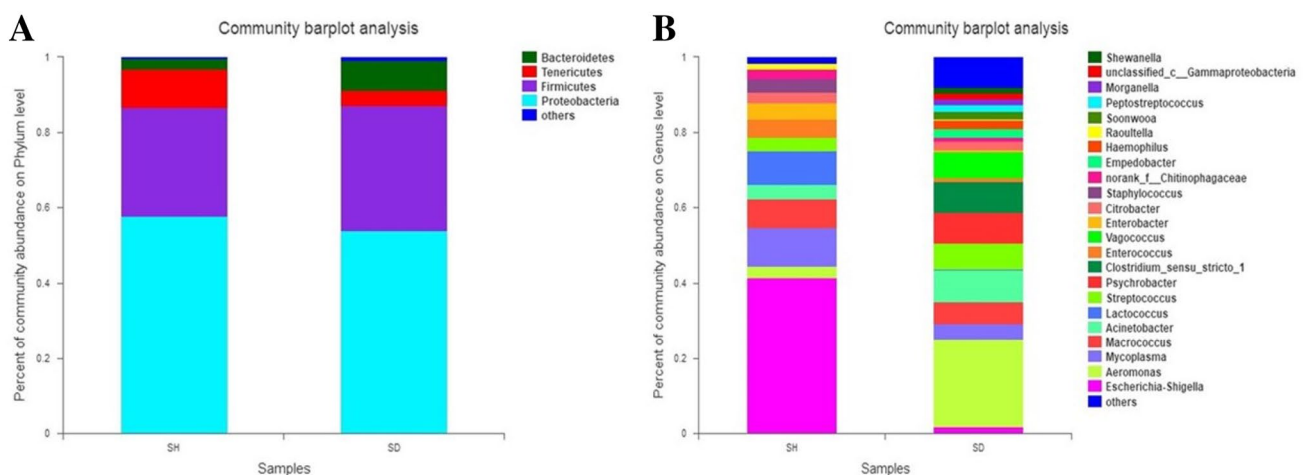


Fig. 4 Lung bacterial community at the phylum (a) and genus (b) levels. Relative abundance of bacterial groups in the lung microbiota. Less than 1% abundance of the phyla or genus was merged into oth-

ers. The swine lung microbial community of the two groups at the phylum level were similar; however, the microbial composition between the two groups at the genus level differed obviously

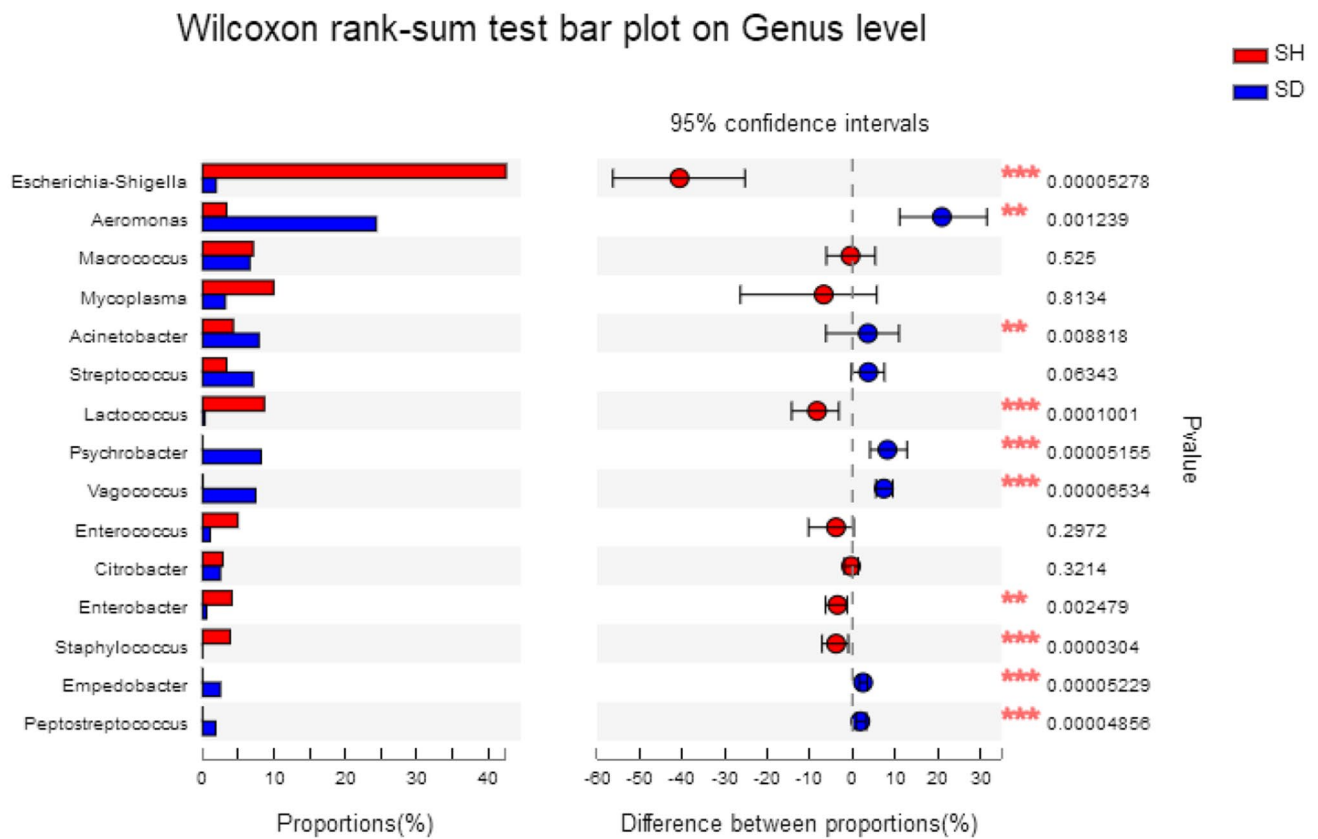


Fig. 5 Comparisons of the relative abundance at the level of bacterial genus in SH group and SD group. The ordinate indicates the bacterial name at genus levels, and the abscissa indicates the percentage value of a bacterial abundance of the sample

Discussion

The lung microbiota inhabiting the lower respiratory tract play important roles in maintaining lung health by modulating the communities of swine lung microbiota. The growth performance and systematic immunity of swine can be regulated during respiratory infection. Studies on the associations between respiratory microbial communities and respiratory tract health provide ample knowledge of host–microbiome interactions (Moffatt and Cookson 2017). During the past decade, culture-independent sequencing techniques have helped demonstrate that the diverse communities of lung microbes play different roles in healthy and disease states. However, the biological and clinical characteristics of these lung microbes remain to be determined (Dickson et al. 2013, 2016). Large quantities of evidence indicated that bi-directional crosstalk between the lung microbiome and the host immune system had an important impact on the occurrence of pulmonary diseases. Microbiome-derived metabolites might shape the lung immunological landscape. For example, LPS, recognized by TLR4 in the lower respiratory tract cells, could up-regulate the expression of various pro-inflammatory chemokines and adhesion molecules, while

short-chain fatty acids (SCFAs) could improve pathogen clearance and protect against viral infection (Zheng et al. 2020).

In this study, a total of 552 qualified OTUs (97% sequence similarity) were identified after low-quality reads were removed. The number of OTUs in the SD group (431) was much higher than in the SH group (296), indicating that samples of the SD group contained many more microbial species (Fig. 1). Alpha diversity analysis performed by community indices (Shannon and Simpson) also showed that the bacterial diversity of SD group was significantly higher than that of the SH group, while Chao1 and Ace indices revealed that the bacterial richness of the SD group was significantly higher than that of the SH group (Fig. 2). A previous study demonstrated that the change of microbiome diversity and composition played an important role in pigs in response to virus infection (such as PRRSV and PCV2) (Ober et al. 2017). In the current study, the predominant bacterial taxonomic compositions were analyzed at the phylum and genus levels, respectively, where Proteobacteria, Firmicutes, Tenericutes, and Bacteroidetes were verified to be the most predominant phyla (Fig. 4a), while *Escherichia-Shigella*, *Aeromonas*, *Mycoplasma*, *Macrococcus*,

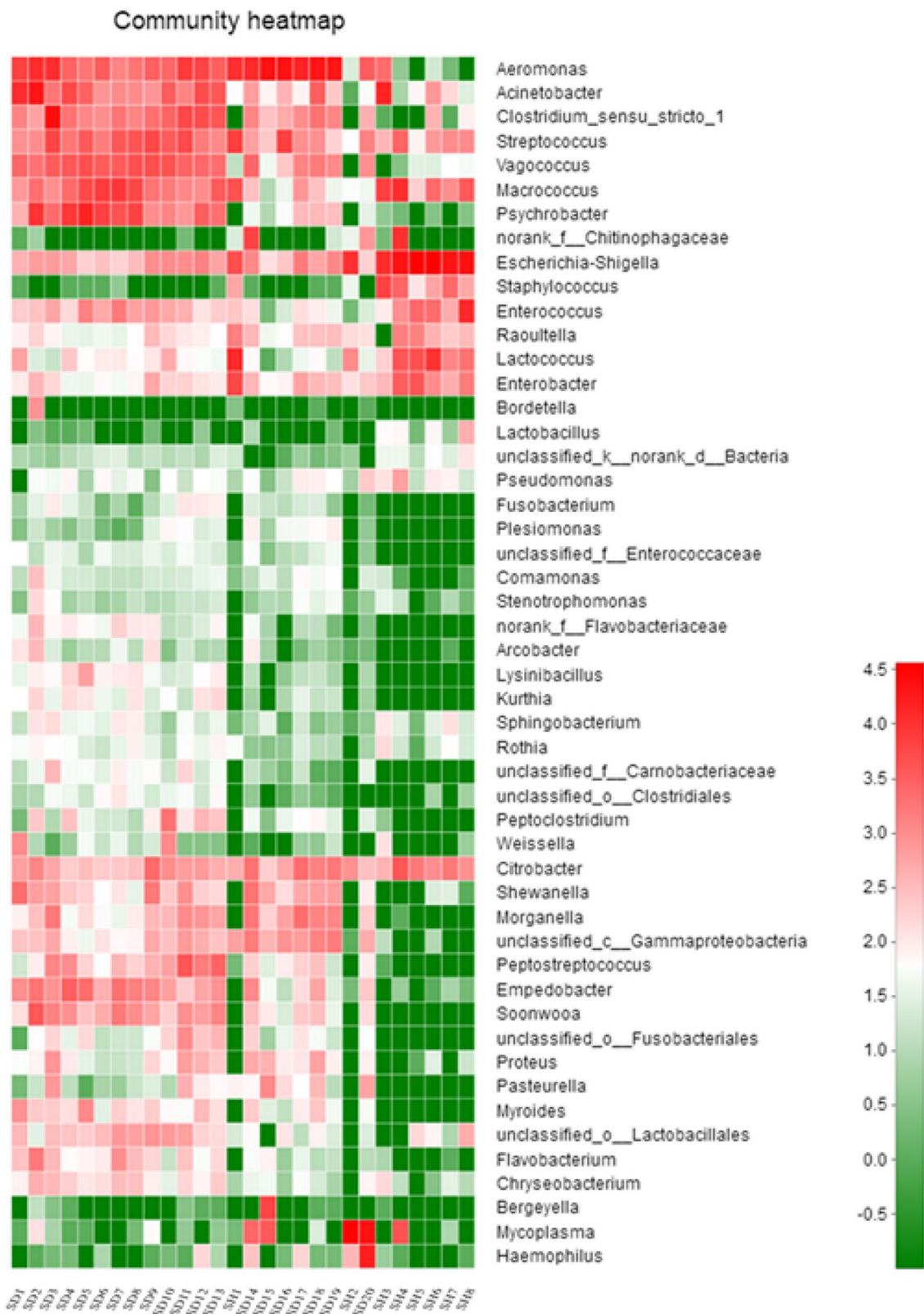


Fig. 6 Heatmap of hierarchy cluster results for the abundance of genus. The color of the spots corresponded to the normalized and log-transformed relative abundance of the OTUs. The genus names of the OTUs are shown on the right

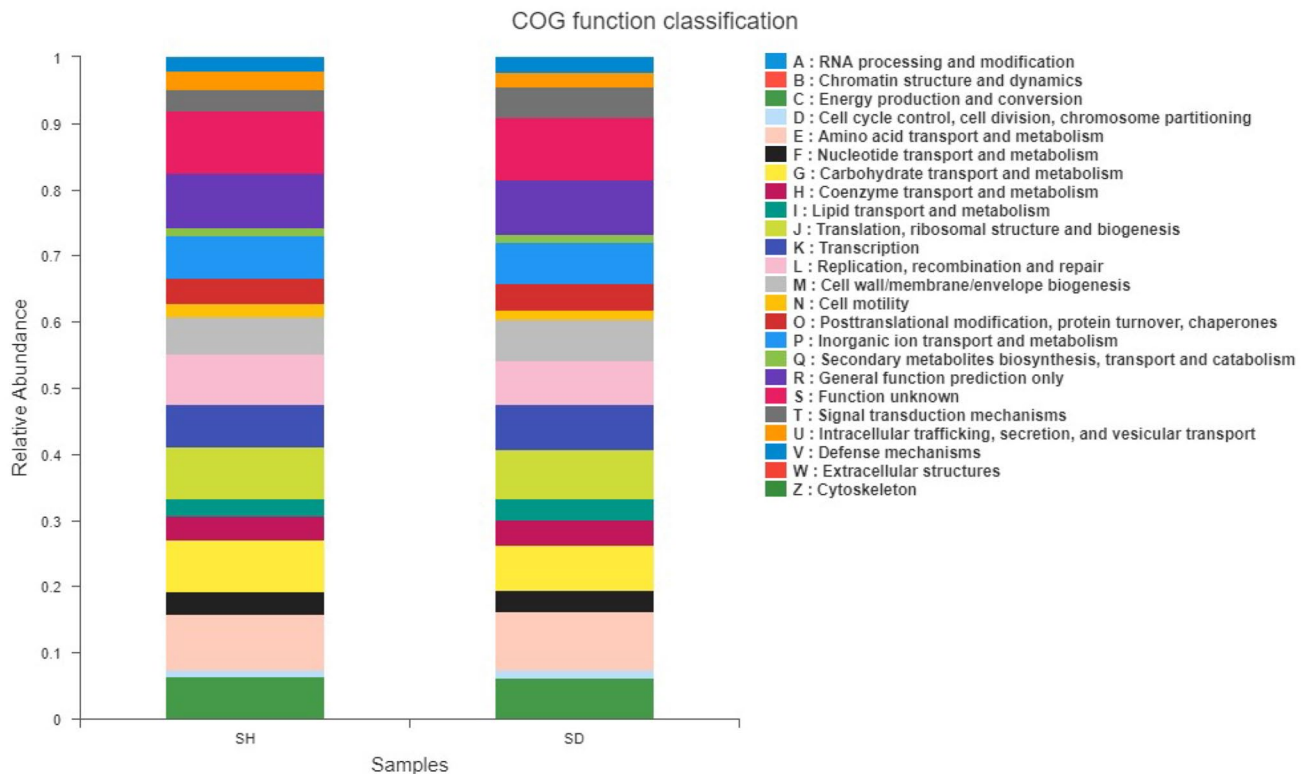


Fig. 7 PICRUSt functional prediction was performed using EggNOG database; more than 23 pathways related to swine respiratory diseases were identified

Acinetobacter, *Lactococcus*, *Streptococcus*, *Psychrobacter*, *Enterococcus*, and *Vagococcus* were confirmed to be the top 10 predominant genera (Fig. 4b). Beta diversity analysis using PCoA revealed that swine lung microbiota from two different groups were segregated into different community clusters (Fig. 3). Therefore, the formation and development of the pulmonary microbiome might play an important role in regulating respiratory disease (Slifierz et al. 2015; Wang et al. 2019).

Previous evidence showed that pig microbiome diversity when changed was linked to a significant reduction in morbidity and mortality following co-infection with PRRSV and PCV-2; therefore, the respiratory microbiome could be selected as a novel treatment target for regulating animal growth performance and disease susceptibility (Niederwerder 2018; Niederwerder et al. 2018). Some symbiotic microbes (such as *Lactobacillus*) were found to have protective effects by producing beneficial metabolites, including lactic acid and short-chain fatty acids (Konstantinov et al. 2004). In our study, the richness of *Lactococcus*, *Staphylococcus*, and *Lactobacillus* genera were all clearly higher in the healthy group than in the diseased group, indicating that these probiotics might have potential protective benefits for the host by preventing pathogen invasion. Administering probiotics (such as *Lactobacillus*) might provide effective

strategies to prevent primary viral respiratory infections and secondary bacterial infections by improving the mucosal antiviral innate immunity and reducing immunopathology (Yin et al. 2018). The beneficial impacts induced by respiratory tract probiotics might be associated with the innate immune response activated by Toll-like receptors (Rocha-Ramírez et al. 2017). *Mycoplasma* genera was identified in the two groups. Therefore, eradication of *Mycoplasma* is urgently needed in modern pig production systems for respiratory disease control (Maes et al. 2018; Surendran et al. 2019). Another endemically infected porcine respiratory tract pathogen is *Streptococcus suis*, which can infect both humans and animals and may cause a variety of fatal diseases (such as meningitis and streptococcal toxic shock syndrome) (Li et al. 2019; Hennig-Pauka et al. 2019). In our study, the composition of *Streptococcus* genera was significantly higher in the diseased group than in the healthy group; therefore, control of *Streptococcus suis* is important for PRDC management in the swine industry. *Haemophilus parasuis* can also cause serious problems in the pig upper respiratory tract, usually occurring after influenza virus infection (Mussá et al. 2012). The relative abundance of *Haemophilus* genera identified in the diseased group was obviously higher than in the healthy group. Other bacterial species that can cause swine pneumonia, such as *Pasteurella*

and *Bordetella*, were also found in the diseased group. Therefore, preventing the transmission of these bacterial pathogens can provide efficient control of swine respiratory diseases (Hughes et al. 2018; Xiao et al. 2019).

In conclusion, the diverse microbial communities of lung microbiota in swine bronchoalveolar lavage fluids were identified and investigated, and the interdependent relationships between lung microbiota and the swine respiratory diseases were analyzed. Analyses of lung microbiota communities and their diversity might provide novel treatment strategies to control swine respiratory tract infections.

Funding This work was supported by the Chinese National Natural Science Foundation Grant (31672606).

Data availability Sequence data have been deposited in the GenBank database under the accession number of ID PRJNA615332.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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