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Effect of Sulfur Dioxide Inhalation on Lung Microbiota in Rat Model

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Abstract

Purpose: To investigate the effect of sulfur dioxide on the lung microbiota of healthy rats. Methods Fifteen male rats were randomly divided into high dose and low dose exposure group and control group. After 7 days of SO2 exposure, the lung tissues were obtained and the lung microbiota was identified by Illumina high-throughput sequencing. Results The microbial community of lung microbiota was significantly alternated in the exposure group and the dominant phylum changed from *Firmicutes* to *Proteobacteria*. In addition, the SO2 exposure caused the bronchial wall thickening and a large number of inflammatory cell infiltration in the lungs of rats in exposure groups. Conclusions The results suggest that SO_2 can significantly alter the lung microbiota and pathological structure of the lungs.

Keywords: Sulfur dioxide; Lung microbiota; High-throughput sequencing; Rat.

1. Introduction

As a widely existing gaseous air pollutant, sulfur dioxide (SO_2) could be easily inhaled into the human body, especially in developing countries [1, 2];. SO_2 emissions are mainly produced from industrial processes, coal-burning, and vehicle emissions. It can be readily solubilized upon entering the airways, dissociating into its derivatives, bisulfite and sulfite [3]. Epidemiological studies have shown that increased SO_2 concentration was associated with various respiratory diseases, such as bronchitis [4], asthma [5], COPD [6] and even lung cancer [7]

The healthy lower respiratory tracts, especially lungs, were considered sterile in the traditional view due to the limit of culture-based technique and the contamination of upper respiratory tract during sampling. However, researches in recent years had revealed that a certain number of bacteria colonizing the lungs [8-10]. In our previous study, we identified the microbial community of lung in the healthy rats and asthma rat model by high-throughput sequencing [11]. The results have shown that *Firmicutes* and *Proteobacteria* were dominantly colonized in the lung of healthy rats and lung microbiota between healthy rats and asthmatic rats was significantly different [12]. It demonstrates that lung microbiota is closely associated with the development of lung diseases and inflammation. Therefore, the interaction between SO_2 and lung microbiota has attracted our attention.

2. Materials and Methods

2.1. Animals and Exposure

Fifteen specific pathogen-free male Sprague–Dawley rats were provided by the Experimental Animal Center of Chongqing Medical University [SCXK-(Yu) 2012-0001] with a body mass of 180±20g. The rats were fed under daylight-simulating light for 12 hours, followed by 12 hours of darkness (room temperature 23 ± 2 ℃, relative humidity of 50–70%, natural ventilation). The animals had access to clean food and water *ad libitum*.

The 15 rats were randomly divided into three groups with 5 rats in each group: low-dose exposure group(group A), the rats were exposed to 5 parts per million (p.p.m., 13.1mg/m3) SO2; high-dose exposure group (group B) , the rats were exposed to 10 parts per million (p.p.m., 26.2mg/m3) SO2; and control group (group C), the rats were exposed to medical-grade air. Rats of exposure group were continuously exposed in a poisoning chamber with a volume of 60 liters (Hope Co, Tianjin, China) for 1 h daily. The exposure dose setting was based on the data of Yargicoglu P and Amamda L's researches in which 5-10 p.p.m. was selected to mimic urban atmospheres [13, 14].

2.2. Sample Collection

Three rats of each group were selected randomly and sacrificed on 7th day. Rats were dissected under sterile conditions. The lower lobe of the left lung was harvested and placed into the liquid nitrogen immediately. Thereafter, they were transferred to −80 ℃ refrigerator within one hour. The low-dose exposure group samples were labelled as A1–A3, the high-dose exposure group samples were labelled as B1–B3 and the control group samples were labelled as C1–C3. The lower lobe of the right lung was harvested at the same time for pathological examination.

2.3. H and E Staining

The lung tissues were fixed in 10% formalin solution, embedded in paraffin, sectioned at a thickness of 3 μm, and stained with H&E. Afterward, the pathological changes in the lung tissues were observed under a light microscope.

2.4. DNA Extraction and Illumina High-Throughput Sequencing

The microbial DNA was extracted from lung samples using the TansStart FastPfu DNA kit (Transgene Biotech, Beijing China) in accordance with the manufacturer's protocols. The V4–V5 regions of the bacteria 16S ribosomal RNA gene were amplified by PCR (95 °C for 5 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for10 min) using primers 515F 5'-barcode-GTGCCAGCMGCCGCGG)-3' and 907R 5'-CCGTCAATTCMTTTRAG TTT-3', where the barcode is an eight-base sequence unique to each sample.

The Illumina high-throughput sequencing was performed by Shanghai Meiji Biomedical Technology Co., Ltd. The purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina HiSeq platform in accordance with the standard protocols. The operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (http://drive5.com/uparse/). The chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analysed by the RDP classifier (http://rdp.cme.msu.edu/) against the silva (SSU115)16S rRNA database using a confidence threshold of 70%.

2.5. Statistical Analysis

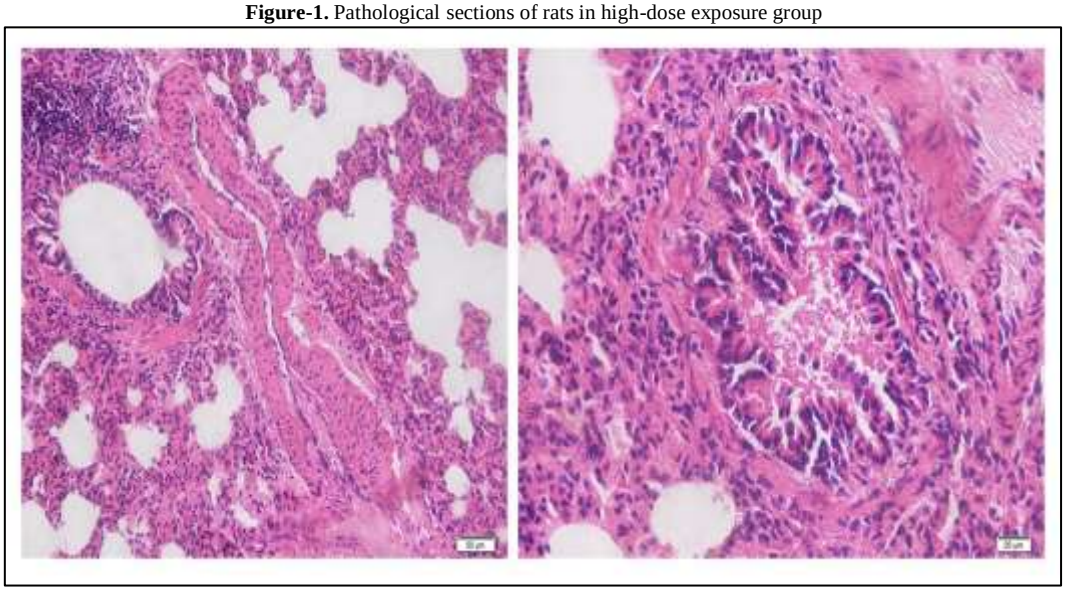
Ordinary one-way ANOVA with the Student–Newman–Keuls test (P<0.05) was performed to investigate the significant differences of each group using SPSS software (IBM, version 20). A p-value < 0.05 was considered statistically significant。

3. Results

3.1. Pathological Examination

Compared with those of the control group, the pathological sections of the exposure groups showed that SO2 exposure caused bronchial wall thickening (Figure 1, left) and a large number of neutrophils, inflammatory cell infiltration (Figure 1, right). It demonstrated that SO2 inhalation could destroy bronchial epithelium and cause inflammation of the lung tissues.

Figure-1. Pathological sections of rats in high-dose exposure group



3.2. Alpha-Diversity Analysis

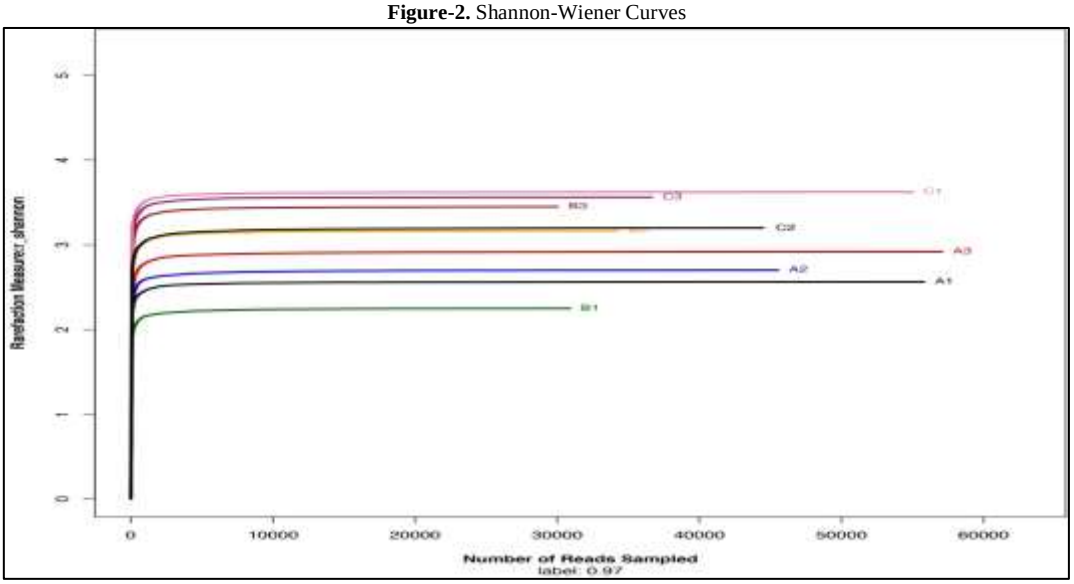
A total of 142,022 microbial sequences and 53,277,926 base pairs (bp) with average length 375.1633333 were detected in low dose exposure group, 140,804 sequences and 52,784,725 base pairs (bp) with average length 374.8733333 were detected in high dose exposure group, and 125,399 sequences and 47,006,853 base pairs (bp) with average length 374.86 were detected in control group.

The Chao index, Shannon index, Simpson index and the Coverage index for each sample are shown in Table 1. The coverage index was determined to reflect whether the sequencing results represented the true situation of the microbiome in the samples. The ANOVA results showed the difference in Chao index was statistically significant (P_{Chao} <0.01), but there were no statistically significant differences in Shannon and Simpson indexes among groups ($P_{Shannon}$=0.648, $P_{Simpson}$=0.495). The Chao index was used to reflect the community richness of the microbiome. The higher the Chao index was, the more abundant the microbiome would be.

Table-1. Analysis of community diversity

Sample ID	Reads	0.97				
		OTU	Chao	Coverage	Shannon	Simpson
A1	54192	292	281	0.999800	3.62	0.0713
A2	48964	243	290	0.999757	3.85	0.0496
A3	46346	242	289	0.999563	3.56	0.0738
B1	47423	291	286	0.999779	3.88	0.0452
B2	46826	372	371	0.999512	4.03	0.0416
B3	52158	348	362	0.999826	4.16	0.0384
C1	47745	719	741	0.998419	4.85	0.0216
C2	45821	621	700	0.996801	3.93	0.0585
C3	48261	674	604	0.997728	3.21	0.0986

Figure-2. Shannon-Wiener Curves



The Shannon–Wiener curves generated by MOTHUR plotting the number of reads using the Shannon index tended to approach the saturation plateau (Figure 2). All of the curves were flat, indicating that the sequencing depth was sufficient to cover more than 97% of the microbiome in the samples.

3.3. Taxonomic Composition

A total of 19 phyla were identified from all samples. The microbial community of all samples in phylum level were displayed in Figure 3. In three control group samples the most dominant phylum was *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria*, which represented over 95% of the total abundance. *Firmicutes* was replaced by *Proteobacteria* in all the exposure samples (Figure 3).

Figure-3. Microbial community compositions at phylum level

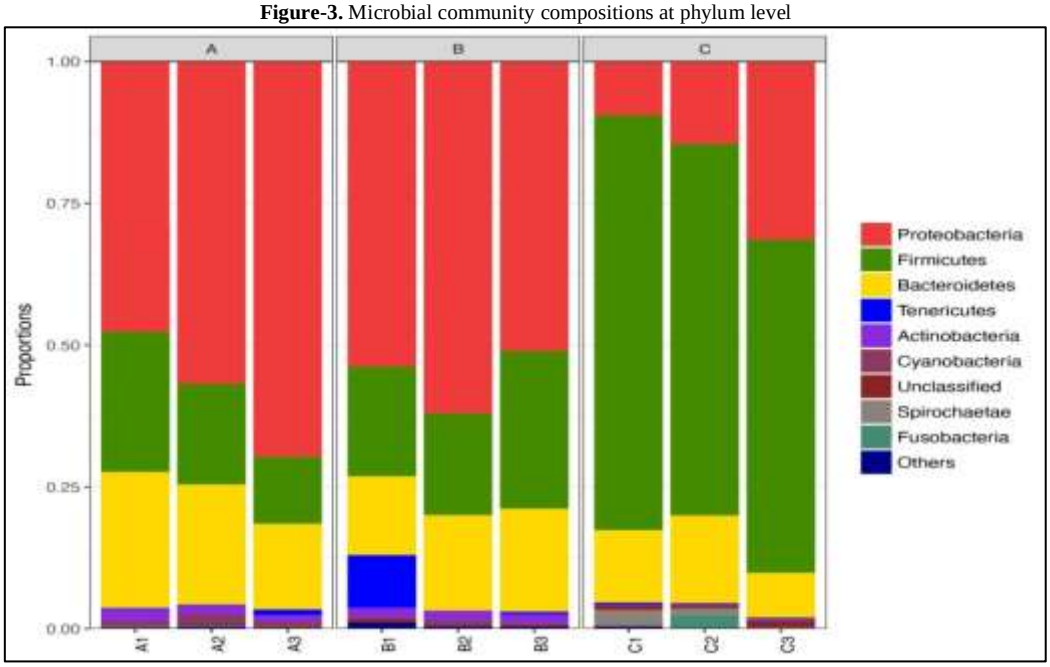
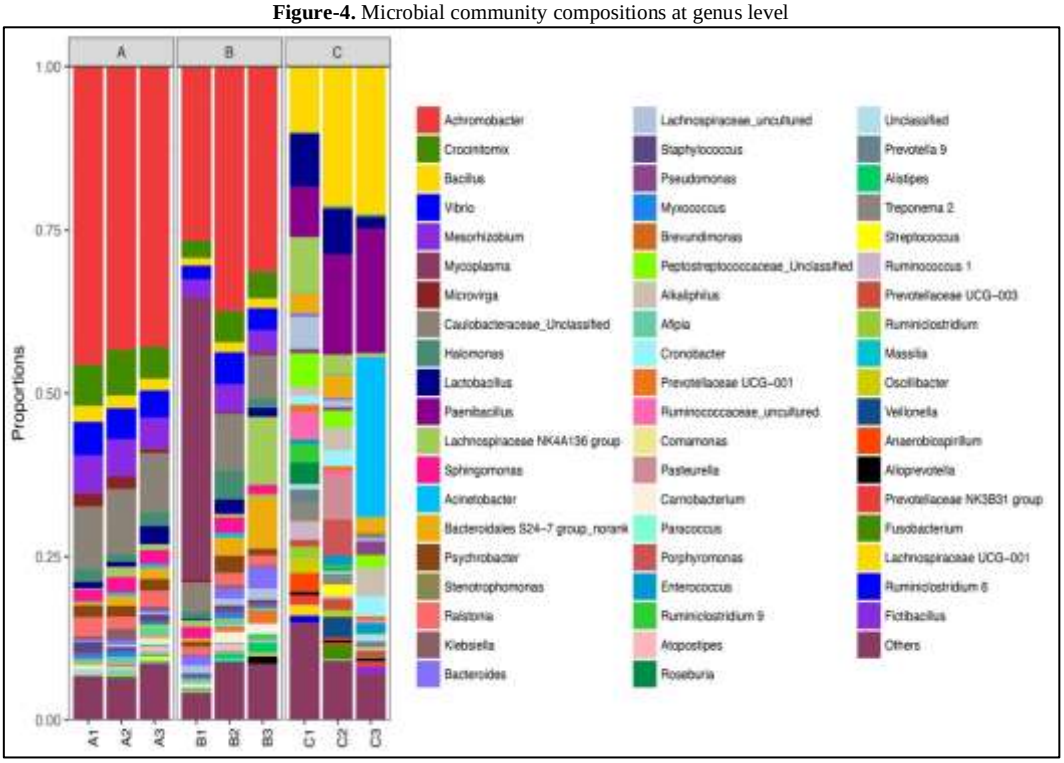


Figure-4. Microbial community compositions at genus level

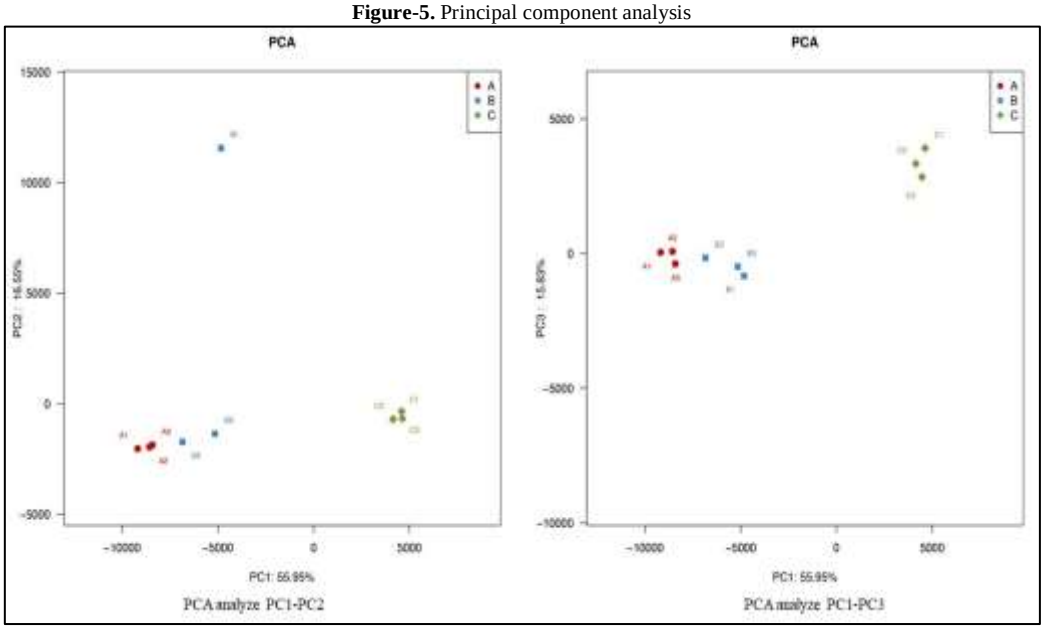


In genus level, the top 3 dominant members in control group were *bacillus*, *paenibacillus* and *lactobacillus*, which were the same as our previous research results. In the low dose exposure group, *achromobacter* was the most dominant taxon which relative abundance in each sample accounted for almost half of the proportions. In the high dose exposure group, *achromobacter* was still the most dominant taxon but the proportions were slightly decreased. It was worth noting that the relative abundance of *mycoplasma* was abnormally high in sample B1. It might be caused by contamination during the sampling procedure.

3.4. Differential Analysis

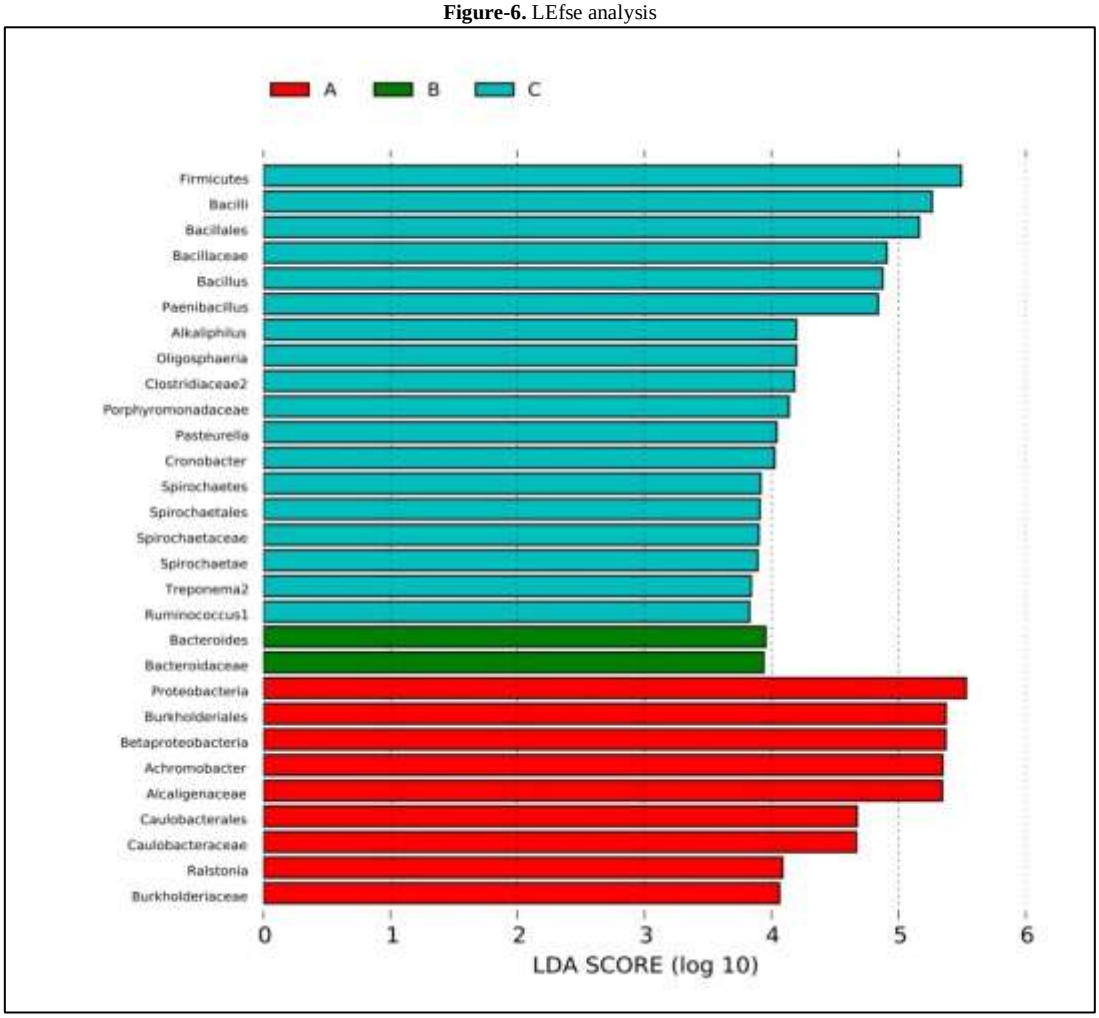
Principal component analysis (PCA) was performed to assess the overall correlation of the microbial populations presented in all the samples (Figure 5). When all the samples were included in the analysis, principal component 1 (PC1) demonstrated a complete separation of the control group samples from the exposure group samples. The sample B1 was separated from other samples due to the contamination of *mycoplasma.*

Figure-5. Principal component analysis



The linear discriminant analysis effect size analyses (Lefse) were conducted to identify the key taxa responsible for the differences among the samples. The linear discriminant analysis (LDA, Figure 6) showed differences in relative abundance among the three groups. The most unique taxa in the control group were *Firmicutes*, *Bacilli*, and *Bacillales*, and those in the exposure group were *Proteobacteria*, *Burkholderiales*, *Betaproteobacteria*, *Bacteroides*, and *Bacteroidaceae*.

Figure-6. LEfse analysis



4. Discuss

The lower respiratory tracts, especially the bronchioles and lungs, had been considered to be sterile for decades until the concept of lung microbiota was proposed by Hilty, *et al.* [8]. In our previous research, we identified the colonization of a large number of bacteria in healthy rat lungs by high-throughput sequencing [11]. Further research showed that lung microbiota between healthy rats and asthmatic rats was significantly different [12]. These results suggested that the lung microbiota was closely associated with the development of lung diseases and inflammation. The microbial community of respiratory tract could be easily affected by many air toxic factors such as smoking, particulate matter, so we designed a series of experiments to explore the interaction between external air pollution factors and lung microbiota in rat model.

SO2 is one of the major air pollutants which is easily inhaled into the respiratory tract. Sulfurous acid and sulfuric acid, two kinds of its derivatives, are strong irritants and can directly cause the burning effects on the respiratory tract immediately. Van Keymeulen, *et al.* [15], reported that 30 minutes of sulfur dioxide treatment would change the ultrastructure of the airway epidermis of guinea pigs. In the current study, we also observed that the structure of bronchus was significantly changed by SO2 exposure. The bronchus walls thickened and a large number of inflammatory cells infiltrated into bronchial layers. This observation was consistent with the other reports [14]. The microenvironment within the tissue was the foundation of the microbial populations, and the change of the organization will inevitably lead to the change of the microbial community. On the other hand, the alternation of the microbial community would adversely react with the lung tissue, further aggravating the damaged tissues.

Further analysis of the high-throughput sequencing data showed that the structure of the microbial community in the lungs of the rats in exposure groups was significantly different from that in control group. The most dominant phylum in the lung microbiota of the rats in exposure groups was *Proteobacteria*, comparing to *Firmicutes* in control group. According to the published reports and our previous research, *Firmicutes* was the most dominant phylum both in healthy human and rat [16]. In genus level, the most dominant genus changed from *bacillus* to *achromobacter*. The alteration of the microbial community in lungs would lead to the potential pathogenic pathogens colonization and played an important role in the development of respiratory diseases and inflammation [17].

5. Conclusion

As one of the major air pollutants, SO2 was associated with many respiratory diseases and inflammation. In this study, we found that SO2 exposure can alter the microbial diversities and communities of lung microbiota in rats.

The microbial community is associated with the state of human health and the occurrence and development of diseases. It might play an important role in respiratory diseases caused by SO2.

Acknowledgments

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