Exposure to traffic-related PM2.5 pollutants significantly affect the diversity and quantity of lung microbiota in a rat model

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Abstract. To investigate the microbial alteration of the lung in rats after the traffic-related PM2.5 exposure, thirty female rats had exposed to traffic-related PM2.5 and normal air for 17 days, the microbial changes were identified by Illumina high-throughput sequencing. The pathological structure and the microbial diversity of lungs in exposure groups were significantly changed. The most dominant phylum in lung microbiota changed from Firmicutes to Proteobacteria. Also, Sphingomonas was identified as the key differential bacterium between the exposure and control groups. The results suggest that traffic-related PM2.5 can affect the distribution of lung microbiota and may cause damage of the lung tissues and function.

1. Introduction
Fine PM or PM2.5, which refers to particles with an aerodynamic diameter of 2.5 μm or less, has been extensively investigated due to the large-area and long-haze weather in China [1]. Epidemiological studies have shown that PM2.5 is associated with various respiratory diseases, such as abnormal lung function [2], lung infection [3], asthma [4], and lung cancer [5]. In Wu’s study [6], the land regression model was established to evaluate the concentration distribution of PM2.5 in Chongqing. The results showed that PM2.5 concentration along the primary highway in Chongqing was higher than in other areas. Therefore, traffic-related PM2.5 was the primary source of PM2.5 pollution. In our previous research, Proteobacteria and Firmicutes were dominantly colonized in the lung of healthy rats [7]. Further research found that lung microbiota between healthy rats and asthmatic rats was significantly different [8]. As a carrier of harmful substances, PM2.5 could cause upper respiratory tract flora disorder to colonize pathogens easily [9]. Hence, PM2.5 might have the same effect on lung
microbiota. In the present study, traffic-related PM2.5 samples from Chongqing were collected to study the effects of traffic-related PM2.5 on lung microbiota of rats.

2. Materials and methods

2.1. PM2.5 collection and suspension preparation
The traffic-related PM2.5 was collected continually from 15th June 2016 to 28th February 2017 at three locations near main road and traffic junctions in Chongqing using Intelligent flow air samplers (Tianhong Co, Wuhan China) and PM2.5 cutter and glass fibre filters (d=1 μm, Whatman, Germany). The sampling condition is as follows: air intake flow of 1m$^3$/min, 24-h continuous sampling, daily replacement of the filter, and suspension of sampling when raining. According to the data of China’s air quality monitoring and analysis platform, the highest concentration of PM2.5 during sampling time in Chongqing was 88 μg/m$^3$, the lowest was 22 μg/m$^3$, and the average was 40.8 μg/m$^3$.

The filters were weighed and cut into 1 cm$\times$1 cm pieces. Thereafter, they were ultrasonic washed in 100-mL ultra-pure water for 60 min at 4°C. They were then filtered using an eight-layer gauze. The filtrate was centrifuged at 4°C and 12, 000 r/min for 30 min. The supernatants were abandoned. Subsequently, the precipitate was freeze-dried in vacuum for 24–48 h and lyophilized to obtain PM2.5, the gray-black floc. After the UV irradiation for 0.5 h, the PM2.5 powder was configured to 20 mg/mL of the stained mother liquor with sterilized phosphate buffer saline and stored at 4°C.

2.2. Animals and grouping
Thirty specific pathogen-free female Sprague–Dawley rats without mating were purchased from the Experimental Animal Center of Chongqing Medical University [SCXK-(Yu) 2018-0003] with a body mass of 180±20g. The condition of feeding environments was room temperature 23 ± 2°C, the relative humidity of 50–70%, natural ventilation, with daylight-simulating light for 12 hours, followed by 12 hours of darkness. The animals had free access to clean food and water.

The 30 rats were randomly divided into three groups with ten rats in each group: the control group (group A), low-dose exposure group (group B), and high-dose exposure group (group C). All the rats were continuously exposed in the body poisoning cabinet (Hope Co, Tianjin, China) 2 h a day at 14:00–16:00 for 17 days. The exposure dose setting was based on the data of China’s air quality monitoring and analysis platform and Stephen H. Gavett’s research [10]. PM2.5 suspensions in the low- and high-dose groups were 0.1 and 0.3 mg/ml, respectively. The suspension was ultrasonically atomized into a 600 L poisoning cabinet with a speed of 2.5 ml/min and air intake flow of 1 m$^3$/min.

2.3. Observation and sample collection
The rats were weighed before and after the exposure procedure, every day and the weights were recorded. During the exposure period, the mental condition, toxic reaction, diet, appearance change, and death of the 30 rats were observed closely. The 30 rats were sacrificed on the 17th day and dissected under sterile conditions. The lower lobe of the left lung was harvested, and its surface was washed with sterile saline. The samples were placed into the liquid nitrogen immediately to quickly freeze them. Thereafter, they were transferred to −80°C refrigerator within one hour. The control group samples were labelled as A1–A4, the low-dose group samples were labelled as B1–B4, and the high-dose group samples were labelled as C1–C4. The lower lobe of the right lung was harvested at the same time for pathological examination.

2.4. H&E staining
The lung tissues were put into 10% formalin solution for 24 hours. After embedding in paraffin, the lung tissues were sectioned at a thickness of 3 μm and stained with H&E. The pathological changes were observed under a light microscope.
2.5. DNA extraction and Illumina high-throughput sequencing

TansStart FastPfu DNA kit (Transgene Biotech, Beijing China) was used to extract the microbial DNA following 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 for 10 min) using primers 515F 5'-barcode-GTGCCAGCMGCGG)-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. Raw Illumina fastq files were optimized and filtered. Operational taxonomic units (OTUs) were analyzed using Usearch (vision 7.1 http://drive5.com/uparse/).

2.6. Statistical analysis

Mean and standard error of all data were calculated by ordinary one-way ANOVA with the Student–Newman–Keuls test (P<0.05) using SPSS software (IBM, version 20.0).

3. Results

3.1. Behaviour, appearance, and growth

All rats survived after 17 days of treatment with traffic-related PM2.5. Compared to the rats in control group, the rats in both exposure groups manifested significant abnormal behaviors like restlessness, stress and biting. Food-intake decreased significantly in both exposure groups. As such, the weight of the rats in both exposure groups increased slowly (Figure 1). The results showed that traffic-related PM2.5 inhalation in both exposure doses could have affected the growth and development of rats and caused the abnormalities of the nervous system.

![Figure 1. The body-weight curve](image)

![Figure 2. Shannon–wiener curves](image)

3.2. Alpha-diversity analysis

The main parameters of α-diversity analysis of each sample are listed in Table 1. The ANOVA results showed the differences were statistically significant in Chao, Shannon and Simpson indexes (Pchao<0.01; Pshannon<0.01; Psimpson<0.01). The bacterial abundance and biodiversity of lung microbiota were significantly reduced after the traffic-related PM2.5 exposure. The Shannon–Wiener curves generated by MOTHUR plotting the number of reads using the Shannon index tended to approach the saturation plateau (Figure 2).
3.3. Taxonomic composition
A total of 21 phyla were identified from the control group, with eight phyla showing a relative abundance >0.1%. In agreement with our previous research on healthy rats, the four control group samples were dominated by three phyla: *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, which represented over 95% of the total abundance. A total of 17 phyla, which was less than that of the control group, were identified in the two exposure groups. The dominant phylum of the exposure groups was *Proteobacteria*, which had absolute dominance in all exposure samples with the relative abundance from 32.88% to 96.69% (average 71.54%) (Figure 3). The top three dominant genera of sample A1–A3 were *Bacillus*, *Paenibacillus*, and *Lactobacillus*. The diversity of the exposure groups in the genus level was decreased after the traffic-related PM2.5 inhalation. The dominant genera of both exposure groups were *Sphingomonas*, *Methylobacterium*, and *Caulobacter* (Figure 4).

3.4. Differential analysis
Principal component analysis (PCA) was performed to assess the overall correlation of the bacterial populations present at all the samples. When all the samples were included in the analysis, principal component 1 (PC1) demonstrated a complete separation of the control group samples from the

| Sample ID | Reads | OTU | Chao | Coverage | Shannon | Simpson |
|-----------|-------|-----|------|----------|---------|---------|
| A1        | 43725 | 690 | 729  | 0.998468 | 4.87    | 0.0213  |
| A2        | 35362 | 605 | 622  | 0.997314 | 3.97    | 0.0577  |
| A3        | 44474 | 516 | 604  | 0.997774 | 3.24    | 0.0975  |
| A4        | 43918 | 427 | 446  | 0.999180 | 3.77    | 0.0763  |
| B1        | 29843 | 86  | 91   | 0.999180 | 1.72    | 0.2882  |
| B2        | 29763 | 108 | 114  | 0.999664 | 2.04    | 0.2331  |
| B3        | 29676 | 172 | 191  | 0.999023 | 1.85    | 0.3391  |
| B4        | 29711 | 169 | 207  | 0.999664 | 1.29    | 0.4753  |
| C1        | 29366 | 96  | 99   | 0.999762 | 1.83    | 0.355   |
| C2        | 29813 | 96  | 100  | 0.999698 | 1.94    | 0.2676  |
| C3        | 31461 | 135 | 141  | 0.999619 | 1.96    | 0.3417  |
| C4        | 28927 | 261 | 291  | 0.998202 | 2.77    | 0.1461  |

Table 1. Alpha-diversity analysis
exposure group samples (Figure 5). The exposure group samples clustered together on PC3, whereas the control group samples separated along PC3.

The linear discriminant analysis effect size was used to identify the key taxa responsible for the differences among the samples. *Pedomicrobium*, *Tepidimonas*, and *Propionibacteriaceae* were the most prominent phylotypes in the low-dose exposure group. In the high-dose exposure group, *Sphingomonas* of *Alphaproteobacteria* was the most dominant species (Figure 6).

3.5. Pathological examination

Compared with those of the control group, the pathological sections of the exposure groups showed that the lungs had lymphocytes, neutrophils, and other inflammatory cell infiltrations, alveolar stromal hyperplasia, a large number of inflammatory cells, and tissue phagocytic cell infiltration (Figure 7). The results showed that PM2.5 could cause the inflammation of the lung tissues of rats.

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**Figure 5.** Principal component analysis  
**Figure 6.** LEfSe analysis
4. Discussion
In our previous research, we confirmed the colonization of a large number of bacteria in healthy rat lungs, trachea, and bronchial. Lung microbiota is closely associated with the development of lung diseases and inflammation [11]. The lung microbiota composition can be affected by many factors such as eating habits, smoking, and air pollution [12]. PM2.5, a complex mixture of compounds depending on sources, geography, and topography, is associated with multiple diseases especially respiratory diseases like asthma, pneumonia, lung cancer, which causes great harm to public health and economic losses. Numerous epidemiological studies have shown that long-term exposure to traffic air pollution (TRAP) could influence lung function and played an important role in the pathogenesis of respiratory diseases [13]. In the current study, traffic-related PM2.5 exposure had a significant impact on lung microbiota in rats. The diversity and quantity of lung microbiota were significantly decreased after the exposure, and the dominant phylum changed from Firmicutes to Proteobacteria. At the genus level, Sphingomonas and Methyllobacterium became the most dominant genera in the exposure group. Generally, microbial diversity decreases are associated with dysfunction of the normal microbiota and the possibility of increased pathogenic bacteria colonization. It may explain the inflammatory cell infiltrations in the lung tissue of the exposure group.

What we need to highlight is the alteration of Sphingomonas which has a very broad range of metabolic properties for aromatics and can transport and degrade aromatics, such as naphthalene, biphenyl, toluene, xylene, and cresol, in large quantities. Sphingomonas is also a conditional pathogen that is widely distributed in nature because it is isolated from many different land and water habitats, plant root systems, clinical specimens, and other sources [14]. The change in the internal environment of the lung caused by traffic-related PM2.5 exposure could be the main reason of Sphingomonas colonization, but it also might be the defense reaction of lung microbiota. We will conduct in-depth research on this in further research.

5. Summary
As a consequence of urbanization in China, traffic-related PM2.5 has become a dominant source of PM2.5 pollution in urban areas associated with many respiratory diseases and inflammation. In this study, we found that traffic-related PM2.5 exposure can alter the microbial diversities and communities of lung microbiota in rats. The human microbial community was closely related to human health. Therefore, alternations in the lung microbiota may play an important role in respiratory diseases caused by traffic-related PM2.5.
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