

EXPOSURE TO PARTICULATE MATTER 2.5 LEADING TO LUNG MICROBIOME DISORDER AND THE ALLEVIATION EFFECT OF *AURICULARIA AURICULAR-JUDAE* POLYSACCHARIDE

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Abstract

Objectives: The aim of the paper is to explore the role of lung microbiome disorder in lung tissue injury induced by exposure to particulate matter with a maximum diameter of 2.5 μm ($\text{PM}_{2.5}$) and the alleviation effect of *Auricularia auricular-judae* polysaccharide (AAP). **Material and Methods:** Sprague Dawley rats were given $\text{PM}_{2.5}$ suspension at a dose of 20 mg/l twice a week for 8 weeks. Then, 100 mg/kg or 200 mg/kg of AAP was administered to the rats after $\text{PM}_{2.5}$ exposure. The bronchoalveolar lavage fluid (BALF) and lung tissue samples were collected at the end of the experiment. The BALF was meant to detect changes in lung microbiome by 16S sequences and cluster analysis, with the application of the principal component analysis and the partial least squares discriminant analysis. The levels of interferon- γ (IFN- γ), and interleukin (IL)-4, IL-8, and IL-10 in lung tissue were detected by the enzyme-linked immunosorbent assay method. The pathological changes in lung tissue were observed by hematoxylin and eosin staining. **Results:** After $\text{PM}_{2.5}$ exposure, the alveolar septum was widened, and the structures of alveolar walls were destroyed. There was inflammatory cells infiltration in the alveolar space and the interstitial space. Alpha diversity in BALF showed that the Chao1, ACE, Simpson, and Shannon values were increased, and the lung microbiome analysis revealed that the relative abundance of *Firmicutes* and *Clostridium* increased, while the relative abundance of *Bacteroidetes* and *Akkermansia* decreased. The contents of IFN- γ and IL-8 in lung tissue increased while the content of IL-10 decreased. After the administration of AAP, the alveolar structure damage was alleviated, and the interstitial hemorrhage, edema, and inflammatory cells infiltration were reduced. The Chao1 and ACE values decreased, and the taxonomic abundance values of *Akkermansia* were much higher. Simultaneously, the contents of IFN- γ , IL-4, and IL-8 decreased, and the content of IL-10 increased. **Conclusions:** It was found that $\text{PM}_{2.5}$ resulted in lung microbiome disorder, which might lead to the inflammation of lung tissue. It was also revealed that AAP could alleviate the inflammatory damage of lung tissue induced by $\text{PM}_{2.5}$. Int J Occup Med Environ Health. 2022;35(6):651–664

Key words:

$\text{PM}_{2.5}$, inflammatory damage, lung injury, BALF, microbiome disorder, AAP

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INTRODUCTION

Ambient delicate particulate matter with a maximum diameter of $2.5\ \mu\text{m}$ ($\text{PM}_{2.5}$), a kind of suspended particulate matter, is the leading cause of typical atmospheric pollutants and dust haze. Due to the small particle size, $\text{PM}_{2.5}$ can reach the deep part of the respiratory tract with the airflow, and partially also the pulmonary alveolus into the blood circulatory system, which leads to pulmonary inflammatory reaction and oxidative damage [1].

Studies have shown that alveolar macrophages, as a vital defense barrier of the lungs, play an essential role in phagocytosing particulate matter and releasing cytokines [2]. Alveolar macrophages could phagocytose $\text{PM}_{2.5}$, which then release a series of pro-inflammatory factors and cytokines (such as tumor necrosis factor α [TNF- α]) [3]. The pro-inflammatory factors or $\text{PM}_{2.5}$ deposited in the lungs further act on lung epithelial cells, fibroblasts and endothelial cells, leading to the secretion of adhesion molecules and cytokines (such as interleukins [IL]-8 and IL-6) [4]. These adhesion molecules and cytokines play a crucial part in recruiting various inflammatory cells (such as neutrophils, macrophages, monocytes, and polymorphonuclear leukocytes) to aggregate, resulting in inflammation. Previous research has established that $\text{PM}_{2.5}$ can activate CD4^+ T cells to differentiate into multi-functional T effector cells, which secrete various inflammatory factors such as pro-inflammatory factors (IL-6, IL-8, IL-12, interferon- γ [IFN- γ], etc.) and anti-inflammatory cytokines (such as IL-4 and IL-10) [5]. Nevertheless, the specific mechanism of $\text{PM}_{2.5}$, causing inflammatory injury in lung tissue, is still unclear.

Recently, lung microbiome disorder and inflammation have drawn public attention. Previous research has established that the predominant bacteria in lung tissue, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, interact with the local mucosal immune system and are synergistically symbiotic, maintaining immune stability and balance [6]. However, under the influence

of external factors such as environmental pollution and antibiotic abuse, the lung flora might be in disorder and may induce infection of the pathogen; even some strains in the normal flora may turn into conditional pathogens, causing inflammation. Local flora disturbance can make the active CD4^+ T cells differentiate into T effector cells. For instance, Th1 cells can produce pro-inflammatory cytokines (IL-12, IFN- γ , etc.) and then induce an immune response, while Th2 cells mainly induce a humoral immune response that can produce anti-inflammatory cytokines (IL-4, IL-10, etc.) to inhibit inflammation.

After *Clostridium* stimulates the colon, the body secretes inflammatory factors IL-6 and IL-8, causing immune response [7]. In comparison, a beneficial bacterium such as *Lactobacillus* could induce dendritic cells to secrete anti-inflammatory factors such as IL-4 and IL-10, and to control the further development of inflammation [8]. *Akkermansia*, owing to the function of regulating the body's immune response, could activate and regulate T cells, promote IL-4 and IL-10, and inhibit the expression of IFN- γ and TNF- α , thereby reducing the inflammatory response [9]. Therefore, it can be seen that the flora is closely related to inflammatory factors.

Related research has shown that there are a variety of micro-organisms in $\text{PM}_{2.5}$, including bacteria, viruses, and eukaryotic micro-organisms (fungi, microalgae, and protozoa), and so on [10], which are formed by airborne bacteria adherence [11]. When attached to biological pollutants as a "foreign body" into the lung, $\text{PM}_{2.5}$ can cause lung microbiome disorder, as well as specific and nonspecific immune response, leading to lung injury [12]. However, the specific mechanism for this phenomenon has not been reported.

Auricularia auricular-judae polysaccharide (AAP) is purified and extracted from *Auricularia*. The fruit of *Auricularia auricular-judae* is rich in hetero-polysaccharides that consist of a D-glucose residue backbone with various chains of β -1, 3-branch residues, such as mannose, glu-

cose, xylose, and glucuronic acid [13]. It has been proved that AAP has various pharmacological functions in humans and animals, such as anti-inflammatory [14], antibacterial [15], antioxidant [16], antitumor [17] and anti-radiation activity [18]. However, the AAP-induced alleviation effect and mechanism on lung tissue inflammatory injury caused by PM_{2.5} exposure has not been reported. Therefore, the present study aimed to investigate whether PM_{2.5} exposure can lead to the disorder of the lung microbiome and the alleviation effect of AAP.

MATERIAL AND METHODS

The process of PM_{2.5} sample collection and treatment

The medium flow sampler (PM_{2.5} cutter: Da 50 = 2.5±0.2 μm) was used to collect PM_{2.5} in the atmosphere of Tangshan in January–December 2017 with a quartz fiber filter membrane with a diameter of 90 mm. The sampler was placed in the open ventilation place; each sampling time lasted 24 h, and the flow rate was 100 l/min. After sampling, the collected filter membrane was cut into small pieces, and then put into anhydrous ethanol, treated with the ultrasonic oscillator for 30 min. The suspension was filtered with 8 layers of gauze. The filtrate was freeze-dried in a vacuum, weighed, and preserved at -20°C. Inductively coupled plasma mass spectrometry (ICP-MS) was used to detect the content of Pb, Mn, As, Cr, Ni, Se, Sb, Cd, Tl, Be, and Hg in PM_{2.5}, among which Pb and Mn were the most prominent. Ion chromatography was used to detect the concentration of different ions in PM_{2.5}, in which SO₄²⁻, NO₃⁻, Cl⁻, and NH₄⁺ were the most critical components. The particles were thoroughly mixed by ultrasonic shaking for 30 min before use, and the PM_{2.5} suspension of 20 mg/l was prepared with normal saline.

Experimental animals and treatment

All experiments and surgical procedures were conducted following the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved

by the North China University of Science and Technology Animal Ethics Committee (No. 2017111). A total of 32 healthy male Sprague Dawley rats (aged 8 weeks) were maintained under specific pathogen-free conditions and randomly divided into 4 groups (N = 8): the control group, the PM_{2.5} group, the APP-200 (200 mg/kg AAP intervention) group, and the APP-100 (100 mg/kg AAP intervention) group. After the rats were anesthetized with ether, the rats in the PM_{2.5} group, the APP-200 group, and the APP-100 group received 20 mg/l PM_{2.5} suspensions (a dose of 20 mg/kg) as intratracheal instillation twice a week for 8 weeks. Then, the rats were placed upright and rotated so that the PM_{2.5} suspension could be evenly distributed in both lungs. The rats in the control group received the same volume of normal saline. At the same time, the rats in the APP-200 and APP-100 groups were given AAP 200 mg/kg and 100 mg/kg, respectively, by gavage once a day for 8 weeks. The whole experimental period lasted 8 weeks.

Bronchoalveolar lavage (BAL) fluid acquisition

After the last PM_{2.5} administration, all the rats were anesthetized with isoflurane (5%) and sacrificed by bleeding from the abdominal aorta. The right primary bronchus was ligated, and the left bronchus was cannulated. The BAL procedure was performed as follows: 3 aliquots of 10 ml sterile 0.9% NaCl at room temperature were injected and gently aspirated with a syringe; the BAL fluid (BALF) was centrifuged at 4°C, 1000 r/min for 15 min (a centrifugal radius of 7 cm), following which the supernatant was taken and stored in a -80°C refrigerator.

Hematoxylin and eosin staining for pathological changes in lung tissue

After the lungs of the rats were collected, they were fixed in formalin buffer, embedded in paraffin, and stained with Hematoxylin and eosin (HE). Pathological changes in lung tissue were observed under the light microscope.

Then, IFN- γ , IL-4, IL-8, and IL-10 were detected by the enzyme-linked immunosorbent assay (ELISA) method. Lung tissues were taken and homogenized with cold physiological saline. The relevant indicators were determined by IFN- γ , IL-4, IL-8, and IL-10 ELISA kits (purchased from Neobioscience, Shen Zhen, China); the lowest detection limits were 8.0 pg/ml, 15.6 pg/ml, 2.0 pg/ml, and 30.0 pg/ml, respectively. The specific steps were strictly performed following the instructions determined for the kits.

Lung microbiota analysis

After the rats were sacrificed, the lungs were lavaged with sterile saline and stored at -80° . The samples were sent to BBI Life Sciences Corporation (Shanghai, China) under dry ice conditions and subjected to polymerase chain reaction (PCR) amplification and sequencing. A specific primer with a barcode was synthesized for the V4 region of the 16S rRNA gene. Then, PCR amplification was carried out using 520F (AYTGGGYDTAAAGNG) and 802R (TACNVGGGTATCTAATCC) primers. A barcode distinguished the sample sequence, and each sample sequence was subjected to quality control filtration and then to alpha diversity analysis. Alpha diversity analyzes species diversity in a single sample, including the Chao1 index, the Shannon index, and the Simpson diversity index. The principal component analysis was used to identify different bacteria in different groups.

Statistical analysis

Experimental data were expressed in mean \pm standard deviation ($M \pm SD$). Data sets involving >2 groups were assessed by one-way ANOVA followed by the least significant difference t-tests. The principal component analysis was used to spot differentially identified bacteria in different groups. The relationship between the dominant fungi of lung flora and lung injury – in other words, the relationship between the genus of lung flora and the changes of inflammatory factors in lung tissue – was investigated

by cluster analysis, principal component analysis, and partial least squares discriminant analysis. To this end, SPSS v. 22.0 was used and a p-value of 0.05 was considered statistically significant.

RESULTS

Pathological changes in lung tissue in the rats following PM_{2.5} exposure

As shown in Figure 1, in the PM_{2.5} group, the alveolar septum was significantly widened, and the structure of alveolar walls was destroyed. The capillary blood vessels were dilated and congested. There was inflammatory cells infiltration that occurred in the alveolar space and the interstitial space. Meanwhile, in the APP-200 and APP-100 groups, the alveolar structure damage was relatively moderate, while the interstitial hemorrhage, edema, and inflammatory cell infiltration were significantly reduced. The degree of damage in the APP-200 group was lighter than that in the APP-100 group.

Alpha diversity of lung microbiome in the rats following PM_{2.5} exposure

The high-through sequence was used to examine the composition of lung bacteria. A total of 394 016 influential reads were generated in a separate sequencing analysis, and each sample ($N = 3$ for each group) produced $32\,835 \pm 4261$ influential reads. Subsequently, a total of 290 171 sequences were high-quality sequences, and each sample ($N = 3$ for each group) produced $24\,181 \pm 3596$ high-quality sequences. Under this sequencing depth, most diversity was covered (Figure 2).

The alpha diversity index reflected the diversity difference of lung microbiome in each group. Table 1 shows that the Chao1, ACE, Simpson, and Shannon values in the PM_{2.5} exposure groups increased by 70.26%, 89.24%, 22.37%, and 29.00%, respectively, in relation to the control group, and the differences were statistically significant ($p < 0.05$). Compared with the PM_{2.5} group, the Chao1 and

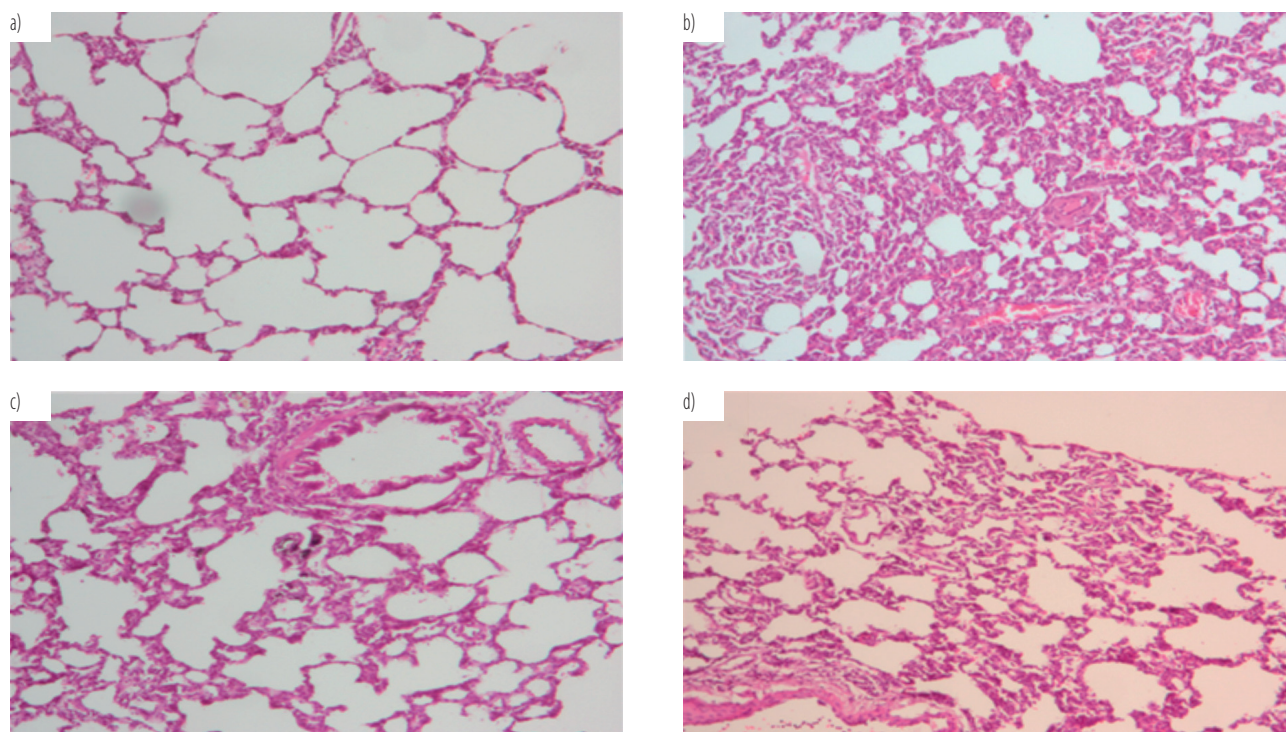


Figure 1. Pathological changes in the lung tissue of rats after exposure to particulate matter with a maximum diameter of 2.5 μm (PM_{2.5}): a) control group, b) PM_{2.5} group, c) auricularia auricular-judae polysaccharide 200 (APP-200) group, and d) auricularia auricular-judae polysaccharide 100 (APP-100) group (hematoxylin-eosin, $\times 100$), in the study on 32 Sprague Dawley rats aimed to investigate both whether PM_{2.5} exposure can lead to the disorder of the lung microbiome and the alleviation effect of AAP in 2017, China

ACE values in the APP-200 and APP-100 groups decreased ($p < 0.05$). All of those were consistent with the study by Poroyko et al. [19].

Beta diversity and the composition analysis of lung microbiome in the rats following PM_{2.5} exposure

The principal component analysis and the partial least squares discriminant analysis (PLS-DA) models were constructed based on the operational taxonomic units (OTU) abundance matrix and sample grouping data. Each point in the figure represented a sample, and points with the same color belonged to the same group. Points of the same group were marked with ellipses (Figure 3a). If some samples had the same characters, they were shown with the same cluster. Figure 3a shows that 4 groups belonged to different

clusters, consistent with the findings by Nguyen et al. [20]. Based on the UniFrac distance, the samples were clustered by similarity (Figure 3b). In summary, the results of this experimental classification model were desirable. Compared with the control group, the microbial community structure of the PM_{2.5} group was significantly different.

Graphlan, a recently emerging visualization tool, was applied to build a hierarchical tree that contained the sample composition at each classification level. Its results illustrated that *Firmicutes*, *Gammaproteobacteria*, *Betaproteobacteria*, and *Bacilli* were the dominant phylum, class, or genus (Figure 4a), similar to literature findings [21].

At the level of the phylum (Table 2), the relative abundance of *Firmicutes* in the lung microbiome of the PM_{2.5} group was significantly higher than that in the control group, while the relative abundance of *Bacteroidetes* was signifi-

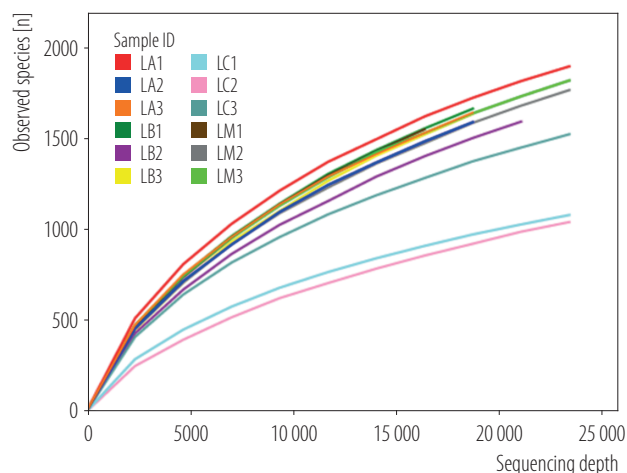


Figure 2. Rarefaction curve – to determine the current sequencing depth of each sample, it is sufficient to reflect the microbial diversity contained in the community sample in the study on 32 Sprague Dawley rats aimed to investigate both whether exposure to particulate matter with a maximum diameter of 2.5 μm ($\text{PM}_{2.5}$) can lead to the disorder of the lung microbiome and the alleviation effect of AAP in 2017, China

cantly lower ($p < 0.05$). Compared with the $\text{PM}_{2.5}$ group, there was no significant change in the relative abundance of *Firmicutes* and the *Bacteroidetes* in the APP-200 and APP-100 groups, which was partly consistent with the results of the Deng et al. study [22].

At the genus level, based on the differences in taxonomic composition among the sample groups (Figure 4b and Table 3), the genus whose abundance was at the top 50 were

drawn (Figure 4c). The relative abundance of *Clostridium*, *Lactococcus*, and *Geobacillus* in the lung flora of the rats exposed to $\text{PM}_{2.5}$ increased significantly, while the relative abundance of *Akkermansia* declined significantly ($p < 0.05$). Interestingly, compared with the $\text{PM}_{2.5}$ group, *Akkermansia*, a potentially beneficial bacterial species, was only increased in the APP-200 and APP-100 groups.

Inflammatory cytokines in lung tissue of the rats following $\text{PM}_{2.5}$ exposure

As shown in Table 4, compared with the control group, the levels of IFN- γ and IL-8 in the lung tissue of the $\text{PM}_{2.5}$ group increased, whereas the level of IL-10 dropped ($p < 0.05$). Compared with the $\text{PM}_{2.5}$ group, the levels of IFN- γ , IL-8 and IL-4 were significantly lower, but IL-10 increased in the APP-200 group, while IL-4 and IL-8 decreased in the APP-100 group ($p < 0.05$), which was partly consistent with the results of the study by Zhuan-Yun et al. [13] and Carneiro et al. [23].

The correlation of lung microbiome and inflammatory cytokines changes in the rats following $\text{PM}_{2.5}$ exposure

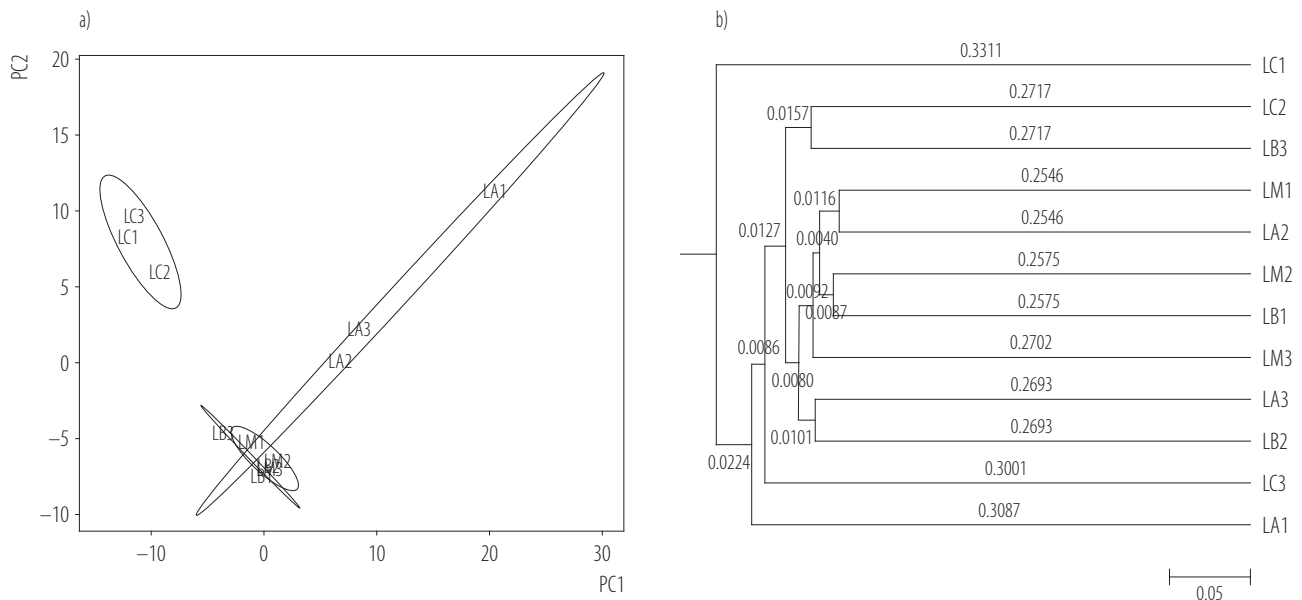
As shown in Table 5, *Firmicutes* were positively correlated with IFN- γ and IL-8, and negatively correlated

Table 1. Alpha diversity of lung microbiome in different groups in the study on 32 Sprague Dawley rats aimed to investigate both whether exposure to particulate matter with a maximum diameter of 2.5 μm ($\text{PM}_{2.5}$) can lead to the disorder of the lung microbiome and the alleviation effect of *Auricularia auricular-judae* polysaccharide (AAP) in 2017, China

Alpha diversity	Group			
	control (N = 3)	$\text{PM}_{2.5}$ (N = 3)	APP-200 (N = 3)	APP-100 (N = 3)
Chao1 (M \pm SD)	575.00 \pm 28.00	979.00 \pm 45.18*	850.67 \pm 24.11**	878.33 \pm 35.57**
ACE (M \pm SD)	743.66 \pm 10.92	1407.28 \pm 8.66*	1119.49 \pm 14.59**	1217.89 \pm 4.57**
Simpson (M \pm SD)	0.76 \pm 0.05	0.93 \pm 0.01*	0.94 \pm 0.02	0.93 \pm 0.01
Shannon (M \pm SD)	4.69 \pm 0.93	6.05 \pm 0.24*	5.93 \pm 0.10	6.19 \pm 0.33

ACE – abundance-based coverage estimation.

* $p < 0.05$ vs. control group; ** $p < 0.05$ vs. $\text{PM}_{2.5}$ group.



PC1 – principal component 1, PC2 – principal component 2.

The bronchoalveolar lavage fluid (BALF) of rats in different groups were examined (N = 3 for each group).

Figure 3. Beta diversity: a) the partial least squares discriminant analysis of lung microbiome in different groups, and b) unweighted pair-group method with arithmetic means clustering analysis based on the UniFrac distance matrix, in the study on 32 Sprague Dawley rats aimed to investigate both whether exposure to particulate matter with a maximum diameter of 2.5 μm (PM_{2.5}) can lead to the disorder of the lung microbiome and the alleviation effect of AAP in 2017, China

with IL-10. *Bacteroides* were negatively correlated with IFN- γ and IL-8, and positively correlated with IL-10. Compared with the control group, the composition of the PM_{2.5} group was significantly different, among which the changes in *Firmicutes* and its *Clostridium*, *Bacteroidetes*, and *Akkermansia* were the most prominent. The IL-8 increase in lung tissue might be related to excessive *Clostridium*. The decrease in the anti-inflammatory factor IL-10 in lung tissue might be due to the decline of *Akkermansia*, resulting in the inflammatory reaction of lung tissue. Compared with the PM_{2.5} group, the bacterial compositions of the APP-200 and APP-100 groups were significantly different in terms of the *Firmicutes*. *Clostridium* decreased, while *Bacteroides* and *Akkermansia* increased, which was related to the decrease in the pro-inflammatory factors IFN- γ and IL-8 in lung tissue and the increased anti-inflammatory factor IL-10.

DISCUSSION

The flora in the air is an essential component of PM_{2.5}. These micro-organisms can diffuse and spread in the suspended particles of the atmosphere [10,11]. After PM_{2.5} enters the body, it might cause a lung-specific and nonspecific immune response, eventually causing lung damage [12]. The current study demonstrated that PM_{2.5} exposure could result in an imbalance of lung flora and inflammation. Meanwhile, AAP could alleviate the inflammation progress.

Data presented by the authors showed that the alveolar septum in lung tissue of the rats following PM_{2.5} exposure was significantly widened, and the structure of alveolar walls was destroyed. There was also inflammatory cell infiltration in the alveolar space and the interstitial space under the microscope, similar to literature findings [24]. These results indicated that inflammatory lesions appear

Table 2. Taxonomic abundance of the lung microbiome in the rats exposed to particulate matter with a maximum diameter of 2.5 µm (PM_{2.5}) with significant differences at the phylum level, in the study on 32 Sprague Dawley rats aimed to investigate both whether PM_{2.5} exposure can lead to the disorder of the lung microbiome and the alleviation effect of *Auricularia auricular-judae* polysaccharide (AAP) in 2017, China

Lung microbiome	Group			
	control (N = 3)	PM _{2.5} (N = 3)	APP	
			APP-200 (N = 3)	APP-100 (N = 3)
<i>Firmicutes</i> phylum [%] (M±SD)	20.15±2.97	34.63±2.75*	34.83±4.57	35.07±0.68
<i>Bacteroidetes</i> phylum [%] (M±SD)	14.03±0.99	6.51±1.24*	7.89±0.78	7.54±0.27

Abbreviation as in Table 1.

* p < 0.05 vs. control group.

Table 3. Taxonomic abundance of the lung microbiome in the rats exposed to particulate matter with a maximum diameter of 2.5 µm (PM_{2.5}) with significant differences at the genus level, in the study on 32 Sprague-Dawley rats aimed to investigate both whether PM_{2.5} exposure can lead to the disorder of the lung microbiome and the alleviation effect of *Auricularia auricular-judae* polysaccharide (AAP) in 2017, China

Lung microbiome	Group			
	control (N = 3)	PM _{2.5} (N = 3)	APP	
			APP-200 (N = 3)	APP-100 (N = 3)
<i>Clostridium</i> genus [%] (M±SD)	0.10±0.02	9.42±3.60	1.97±0.92	0.11±0.03
<i>Lactococcus</i> genus [%] (M±SD)	0.25±0.02*	21.22±0.87*	4.11±0.11*	0.03±0.01*
<i>Geobacillus</i> genus [%] (M±SD)	0.20±0.06	21.19±4.14	4.13±1.07	0.11±0.02**
<i>Akkermansia</i> genus [%] (M±SD)	0.25±0.07	21.50±1.29	4.51±0.50	0.08±0.01**

Abbreviation as in Table 1.

* p < 0.05 vs. control group; ** p < 0.05 vs. PM_{2.5} group.

Table 4. Inflammatory cytokines changes in the lung tissue of rats after exposure to particulate matter with a maximum diameter of 2.5 µm (PM_{2.5}), in the study on 32 Sprague Dawley rats aimed to investigate both whether PM_{2.5} exposure can lead to the disorder of the lung microbiome and the alleviation effect of *Auricularia auricular-judae* polysaccharide (AAP) in 2017, China

Inflammatory cytokine	Group			
	control (N = 8)	PM _{2.5} (N = 8)	APP	
			APP-200 (N = 8)	APP-100 (N = 8)
IFN-γ [pg/mg prot] (M±SD)	13.37±2.62	18.49±3.54*	10.97±1.60**	16.89±1.73
IL [pg/mg prot] (M±SD)				
IL-8	0.68±0.11	3.33±0.78*	0.55±0.11**	0.65±0.10**
IL-4	18.33±3.21	19.83±4.14	14.04±3.40**	15.39±3.86**
IL-10	40.43±6.50	24.89±4.38*	41.10±4.40**	25.63±3.82

IFN-γ – interferon-γ; IL – interleukin.

Other abbreviations as in Table 1.

* p < 0.05 vs. control group; ** p < 0.05 vs. PM_{2.5} group.

Table 5. Correlation between lung microbiome and inflammatory cytokines in the rats exposed to particulate matter with a maximum diameter of 2.5 μm ($\text{PM}_{2.5}$), in the study on 32 Sprague-Dawley rats aimed to investigate both whether $\text{PM}_{2.5}$ exposure can lead to the disorder of the lung microbiome and the alleviation effect of *Auricularia auricular-judae* polysaccharide (AAP) in 2017, China

Lung microbiome	Inflammatory cytokine							
	IFN- γ		interleukin					
	r	p	IL-8		IL-4		IL-10	
r			p	r	p	r	p	
<i>Firmicutes</i>	0.869*	0.025	0.895*	0.016	0.112	0.833	-0.820*	0.046
<i>Bacteroidetes</i>	-0.901*	0.037	-0.994**	0.000	-0.532	0.356	0.926*	0.024
<i>Clostridium</i>	0.598	0.210	0.471	0.345	0.451	0.369	-0.332	0.521
<i>Akkermansia</i>	0.022	0.967	-0.172	0.745	-0.395	0.439	0.104	0.844
<i>Firmicutes/Bacteroidetes</i>	0.918**	0.010	0.993**	0.000	0.411	0.418	-0.834*	0.039

Abbreviations as in Tables 1 and 4.

* $p < 0.05$; ** $p < 0.01$.

in lung tissue after $\text{PM}_{2.5}$ exposure. To prove whether $\text{PM}_{2.5}$ could lead to the disorder in lung flora or not, and to show the relationship between lung microbiome and inflammation, the lung microbiota analysis needs to be performed first.

Alpha diversity of the flora is mainly concerned with the number of species in a localized homogeneous habitat, also referred to the diversity within the habitat. The Chao1 index and the ACE index focus on the richness of the community. Besides, the Shannon index and the Simpson index consider the equitability of the community [25]. The beta diversity analysis of the flora mainly investigated the similarity of the community structure among different samples. The current study used the PLS-DA and cluster analysis based on UniFrac distance to explore the lung flora composition in different groups. Alpha diversity reported in this study showed that the Chao1, ACE, Simpson, and Shannon values of the rats following $\text{PM}_{2.5}$ exposure were increased. Moreover, beta diversity showed that the similarity of the lung flora between the $\text{PM}_{2.5}$ exposed rats and the control group was much lower. These results illustrated that the diversity and structure of the lung flora in rats changed after $\text{PM}_{2.5}$ exposure.

To further investigate the lung microbiome changes in rats, lung microbiome composition at the phylum and genus levels must be analyzed. Previous studies have shown that *Firmicutes* and *Bacteroidetes* are predominant bacteria in lung tissue [6]. Moreover, *Clostridium*, *Lactococcus*, and *Geobacillus* belong to *Firmicutes*. The relative abundance of *Firmicutes* in the lung flora of the rats following $\text{PM}_{2.5}$ exposure increased in the present study, and the relative abundance of *Bacteroidetes* decreased. Meanwhile, the relative abundance of *Clostridium*, *Lactococcus*, and *Geobacillus* in the lung flora after $\text{PM}_{2.5}$ exposure increased, and *Akkermansia* decreased significantly. Notably, $\text{PM}_{2.5}$ exposure leads to the change in the predominant bacteria, but whether this change will result in inflammatory changes in lung tissue requires further study.

As was already noted, $\text{PM}_{2.5}$ can enter the human body through the respiratory tract and be deposited in the respiratory tract or even the alveolus, causing respiratory damage [26]. As regards previous investigations, most microbiological studies related to air flue disease were concentrated on the gut microbiome. Recent studies have shown that even healthy lungs have a unique dynamic

micro population. This conceptual change raises new questions: some lung diseases may have a causal relationship with the ecological imbalance of the local lung flora [27]. Also, as mentioned above, local flora disturbance can make the active CD4⁺ T cells differentiate into T effector cells; whether the changes of certain dominant bacteria caused the inflammation in this study needs further confirmation. As is known, *Clostridium*, belonging to *Firmicutes*, is an anaerobic or microaerobic gram-positive bacterium, which is widely distributed in nature. *Clostridia* can secrete exotoxin or invasive enzymes causing diseases, called *Clostridium* pathogens (*Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, etc.) [28]. *Clostridium* pathogens can stimulate cells to secrete inflammatory factors IL-6, IL-8, causing immune response [7].

What is more, Sverrild et al. [29] has found that the number of *Bacteroides* in the sputum and bronchoscopy biopsy tissue of patients with respiratory tract inflammation was reduced, which suggested the beneficial effect of *Bacteroides*. In addition, the metabolites of *Akkermansia*, metacetic acid and short-chain fatty acid (SCFA) have the property of regulating the body's immune response [30]. *Akkermansia* can also activate and regulate T cells, promote and inhibit the expression of IL-4, IL-10, IFN- γ , and TNF- α , respectively, thereby reducing inflammation [9,13]. The authors of this study detected the content of pro-inflammatory cytokines IFN- γ , IL-8 and anti-inflammatory factors IL-4 and IL-10 in lung tissue, and explored the correlation of lung microbiome and inflammatory factors changes. The presented data showed that the levels of IFN- γ and IL-8 increased, and IL-10 decreased after PM_{2.5} exposure.

Meanwhile, *Firmicutes* were associated and positively correlated with IFN- γ and IL-8, and negatively correlated with IL-10. Besides, *Bacteroidetes* were associated and negatively correlated with IFN- γ and IL-8, and positively correlated with IL-10. It is not hard to find

that the lung flora of the rats was disordered after PM_{2.5} exposure. *Firmicutes* and its *Clostridium* were relatively increased; *Clostridium* secreted more exotoxin or invasive enzymes, which stimulated the body to secrete large amounts of IL-8. Furthermore, due to the relative reduction of *Akkermansia*, the property of regulating the body's immune response to its metabolites (metacetic acid and short-chain fatty acid) was declined. The ability to inhibit the expression of IFN- γ and to promote the expression of IL-10 was weakened. Therefore, ELISA results for detecting inflammatory factors in lung homogenate show that the levels of IFN- γ and IL-8 increased, and the content of IL-10 decreased in lung tissue.

In order to investigate this inflammatory injury, PM_{2.5} exposed rats were treated with 200 or 100 mg/kg of AAP. The obtained data showed that, after AAP treatment, the alveolar structure damage of the lung was mild, as well as the interstitial hemorrhage, edema, and inflammatory cell infiltration were significantly reduced. Meanwhile, the degree of impairment in the AAP-200 group was lighter than that in the AAP-100 group, which is consistent with the finding that AAP attenuates lipopolysaccharide-induced acute lung injury. These phenomena indicate that lung tissue inflammation was reduced in PM_{2.5} exposed rats after AAP intervention. Moreover, it was unexpectedly revealed that, following AAP intervention, not only did the content of IFN- γ and IL-8 in lung tissue decrease, but the content of IL-10 also increased. At the same time, the relative abundance of *Akkermansia* in the lung flora after AAP intervention increased significantly.

Similarly, it can be confirmed that the disorder of the lung flora in rats after AAP treatment improved, *Firmicutes* and its *Clostridium* were relatively decreased, and *Clostridium* secreted less exotoxin or invasive enzymes, which made the secretion of IL-8 reduce relatively. At the same time, due to the relative increase in *Akkermansia*, regulating the body's immune response

for its metabolites was enhanced. The ability to inhibit the expression of IFN- γ and to promote the expression of IL-10 also improved. Therefore, the levels of IFN- γ and IL-8 in lung tissue decreased, while IL-10 increased after the administration of AAP.

Based on the available evidence, the above inferences about the relationship between the lung microbiome and the levels of IFN- γ , IL-8, and IL-10 are relatively reasonable. The changes in IL-4 content in the AAP intervention groups were quite different from those in the IL-10 content. That may be the interference or influence of other factors, which can be confirmed by the fact that there is no apparent correlation between the IL-4 content and the abundance of the flora.

In summary, the mechanism of lung injury after PM_{2.5} exposure is still unclear. This study speculates that the possible mechanism is that PM_{2.5} exposure leads to pulmonary flora disorder, which results in the inflammation of lung tissue. The protective effect of AAP was revealed as regards the inflammatory damage of lung tissue after PM_{2.5} exposure by reducing the levels of IFN- γ and IL-8, and by increasing the IL-10 content.

CONCLUSIONS

It was proved that PM_{2.5} exposure in fact resulted in lung microbiome disorder, which might lead to the inflammation of lung tissue. It was also revealed that AAP can alleviate the inflammatory process in lung tissue induced by PM_{2.5} exposure as well as reduce the levels of IFN- γ and IL-8, and increase the IL-10 content.

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