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Original Research

Can PM2.5 Exposure Change the Expression of ARGs in Intestinal Flora of Wistar Rats?

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Abstract

The mechanism of PM2.5 in the air on health is complicated, in which the gut flora may play a role. The work aimed to study the changes of intestinal microflora in PM2.5 inhaled rats, further, to analyze the expression of antibiotic resistance genes (ARGs) in intestinal flora of the rats. The PM2.5 in the air of Fujian Province of China was collected to establish the rat model of PM2.5 inhaled. The fecal samples were sequenced by metagenomic method. Intestinal flora species annotation, common function database annotation and ARGs were analyzed between PM2.5 inhaled rats and normal control rats. The results showed that the number of Firmicutes was significantly decreased while that of Bacteroides was increased in the PM2.5 inhaled rats compared with the control rats at phylum level. In addition, from genus level to species level, the composition of intestinal flora in PM2.5 inhaled group was totally different from those of control group. Especially, PM2.5 exposure can change the expression of ARGs in intestinal flora. The ARGs of rpoB2, Tet44, TetW and TetO increased significantly in the PM2.5 inhaled Wistar rats. Controlling air pollution and reducing PM2.5 emissions are crucial to maintain public health.

Keywords: PM2.5, intestinal flora, rat, Antibiotic Resistance Genes (ARGs)

Introduction

It was estimated by the World Health Organization (WHO) in 2021 that more than 90% of the world's population is exposed to air pollution, which causes up to 7 million human deaths and affecting the health of 147 million people every year [1-3]. Studies have shown that air pollution (mainly including fine particulate matter PM2.5, ozone and vehicle exhaust, etc.) is one of the most important risk factors for the occurrence of respiratory diseases, cardiovascular diseases, digestive diseases, mental diseases, metabolic diseases and cancer [4-6]. At the same time, the prevalence of ARGs and antibiotic resistant bacteria (ARB) is a serious global public health problem [7]. Due to the overuse of antibiotics, many environments are contaminated by ARGs and ARB, especially including water, soil and air. ARGs belonging to glycopeptides and β-lactam families were the most common in hospital infection, while ARGs belonging to the sulfonamides and tetracyclines families were common in reports from farms, wastewater treatment plants (WWTPs), water

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and soil [7]. The ARGs adsorbed on PM2.5 might spread for a long distance in the atmosphere [7-9].

Studies have shown that the diseases above might be inextricably related to the intestinal flora. The intestinal microbiome is the lifelong community of microorganisms present in the human gut. The total number of the genes encoded by intestinal microbiome is about 150 times than that of by human. It is also known as the second genome of human body [10]. The energy absorption and metabolism of human body are cooperated by the mature and stable intestinal flora, which increases the stability of intestinal mucosa, and promotes the development and maturation of immune system as well [11, 12]. Scholars take the opinions that air pollution could affect intestinal flora, then the intestinal flora could affect various organs and systems on the body in different levels through participating in various metabolic pathways, which may be a risk factor for the occurrence and development of many diseases mentioned above [13, 14]. Therefore, the concepts of "gut-brain" axis, "gut-liver" axis and "gut-lung" axis were proposed [15-17]. In this study, metagenomic sequencing technology [18, 19] was applied to analyze the changes of intestinal microflora and the expression of ARGs in PM2.5 inhaled rats. The research tries to lay a foundation for further studies on the mechanism of PM2.5 in the air on human health.

Materials and Methods

Animal and Group

A total of 36 healthy male Wistar rats with the same age and an average body weight of (150.23±10.10) g were purchased from Shanghai Chang-Ling Experimental Animal Co., LTD., (License Number: SCXK-Shanghai 2018-0003). The rats were adaptively fed with the same diet at ambient temperature of (25±2)ºC, humidity of (60%±5%) and a 12/12-hour light-dark cycle for 4 weeks. From the 5th week, they were divided into PM2.5 exposure group (test, n = 18) and normal control group (control, n = 18).

PM2.5 Exposure Model

HRP-PM286 PM2.5 real-time online concentration and enrichment system (Beijing HuiRongHe Technology Co., Ltd.) was used for the selection and separation of PM2.5 (sample collection location: 450 Dongzhen West Road, Chengxiang District, Putian, Fujian Province, China.). After a concentration (6-10 times) process, the PM2.5 particles were pumped into the box with full animal body exposure (1.2 m^3). The tested rats were exposed for 4h each day (8:00-12: 00) for 21 consecutive days. The concentration of PM2.5 for the exposure was (615.8±439.9) μg/m^3. The tested rats were normally fed daily in the exposure box with clean filtered air after PM2.5 exposure.

Specimen Collection

When the PM2.5 exposure experiment was finished after 21 days, the tails of the rats were lifted, and the lower abdomens of the rats were gently pressed with fingers to induce the defecation reflex. Fresh feces were collected from the anus of the rats with 1.5 mL sterilized EP tube to avoid contamination caused by scratching the fur. All the 36 feces specimens were collected by the same method and put into an incubator with sufficient dry ice when they were sent to the sequencing company for metagenomic sequencing.

Metagenomic Sequencing and Analysis (Functional Analysis Based on Reads)

Total DNA was extracted from the samples. The Illumina NovaSeq high-throughput sequencing platform was used to obtain metagenomic sequences in the fecal samples. The KneadData software was used to control the quality of the original data (based on Trimmomatic) and the de-host sequences (based on Bowtie2). Subsequently, the following three kinds of annotation analyses were carried out: Firstly, species annotation [20]: Kraken2 software and the self-built microbial nucleic acid database were used to compare and calculate the sequence number of species in the samples, then Bracken software was used to estimate the actual abundance of species in the samples. Secondly, abundance cluster analysis [21]: PCoA dimension reduction analysis (species only) and sample cluster analysis were performed based on the information data of species abundance and the information data of functional abundance. Thirdly, annotation of ARGs [22]: Starting from the clean reads removed from the host genes, FMAP software was applied to compare and annotate the quality control of each sample and the reads removed from host genes with CARD database of ARGs (based on DIAMOND), which enabled us to obtain the abundance distribution of ARGs.

Data Analysis

SPSS (version 22.0) was used for the statistical analysis. The normally distributed data was expressed as mean±standard deviation ($\bar{x}$±SD). The comparison between the two groups was performed by independent t test. P<0.05 was considered statistically significant.

Results

Quality Control of Metagenomic Sequencing

All sequencing analysis results in this study were based on the following criterion: The proportion of base numbers with error rate less than 1% (clean Q20) in the sequencing data was above 99%. At the same time,

Table 1. Quality control of sequencing data.

Group	Raw Reads	Raw Base (GB)	Clean Reads	Cleaned (%)	Clean Q20 (%)	Clean Q30 (%)
Control (n = 18)	22187549.33±539694.95	6.65±0.16	21529204.66±512337.20	97.03±0.17	99.15±0.01	96.53±0.01
Test (n = 18)	20643292.01±834140.29	6.19±0.25	19887250.02±841852.89	94.70±2.49	99.13±0.01	96.44±0.05

Note: Raw Reads: the quantity of sequenced Raw reads. Raw Base (GB): the number of Raw reads in GB, the total number of bases in the original data of sequencing, that is, the number of Raw reads multiplied by the sequencing length. Clean Reads: Number of Clean Reads obtained after filtering (quality control and removal of host sequences). Cleaned (%): The percentage of the remaining filtered sequence to Raw reads. Clean Q20: Proportion of bases with a quality score greater than 20. Clean Q30: Proportion of bases with a quality score greater than 30.

that with the error rate less than 1% (clean Q30) in the sequencing data was above 96% (Table 1).

Analysis of Intestinal Microbial Composition

Species Composition Analysis

The reads in the two groups were composed of Bacteria (16511760), Phages (1853), Plant sand Fungi (284). The proportions of these species were 99.99%, 0.01% and close to 0.00%, respectively. The levels of phylum, family, genus and species which was with remarkable characteristics were selected for further analysis and statistics in this study.

Abundance Analysis at Phylum Level

At the phylum level, Bacteroidetes, Proteobacteria, Verrucomicrobia and Ascomycota accounted for the main proportion of intestinal flora in both of the two groups. However, the relative abundance of Firmicutes in the test group was lower than that in the control group, while the relative abundance of Bacteroidetes was higher than that in the control group. To compare the differences between the two groups, the top 20 species in absolute abundance were selected to establish cluster analysis (shown as the clustering heat map at phylum level, Fig. 1).

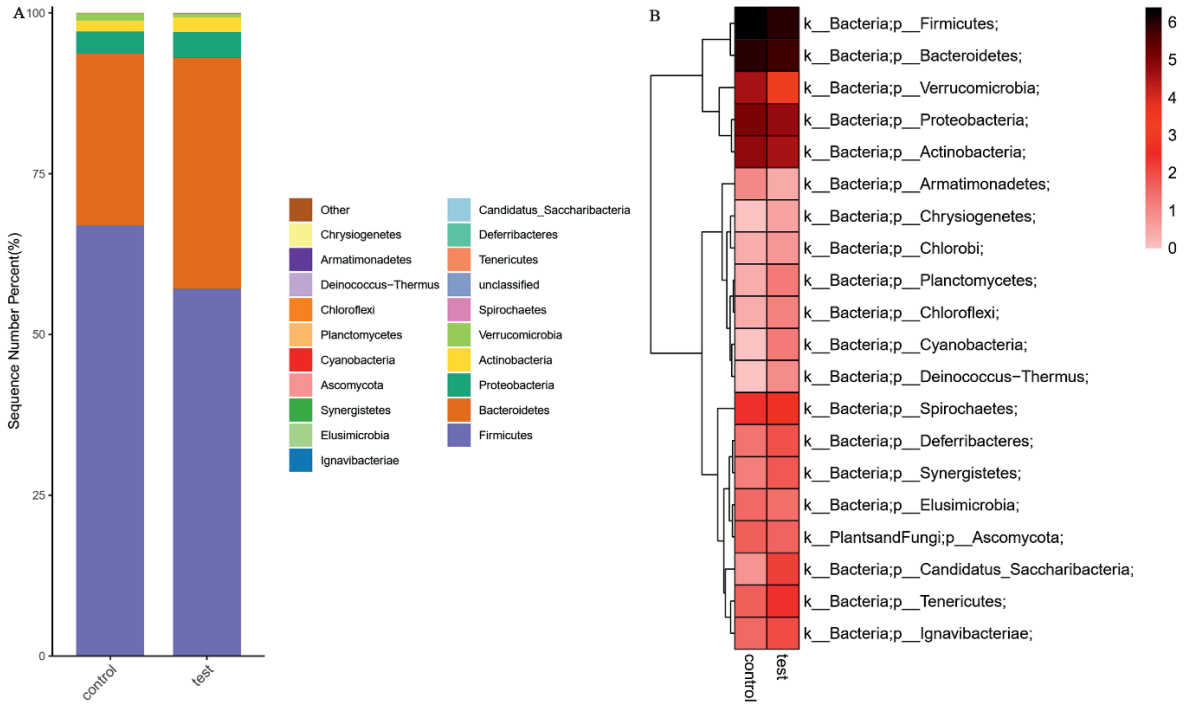

Fig. 1. Relative distribution of species at phylum level. a) Relative distribution analysis at phylum level (top 20 in relative abundance). b) Clustering heat map at phylum level (top 20 in absolute abundance). Note: The top 20 dominant species were shown in Fig. 1a). The remaining species with low relative abundance were classified as Other in the figure. In Fig. 1b), the horizontal axis showed the group information, whereas the vertical axis showed the annotated species names at phylum level. The cluster tree on the left of the figure showed the similarity species distribution in each sample. The colors are displayed by relative abundance, ranging from low (pink) to high (black) shown in the scale at the top of the figure.

Relative Abundance Distribution at Family, Genus and Species Level

At the family level, compared with the control group, the relative abundance of Bifidobacteriaceae (t = 11.468, P = 0.000), Helicobacteraceae (t = 8.210, P = 0.000) and Lactobacillaceae (t = 6.401, P = 0.000) in the test group was significantly decreased, while that of Enterobacteriaceae (t = 4.746, P = 0.000), Lachnospiraceae (t = 7.189, P = 0.000) and Peptostreptococcaceae (t = 7.472, P = 0.000) increased significantly. The relative distribution of the two groups at family level was shown in Fig. 2(a-b).

At the genus level, compared with the control group, the relative abundance of Lactobacillus (t = 6.401, P = 0.000), Helicobacter (t = 8.210, P = 0.000) and Bifidobacterium (t = 11.468, P = 0.000) in the test group was significantly decreased. On the contrary, the relative abundance of Muribaculum (t = 8.681, P = 0.000), Eubacterium (t = 6.359, P = 0.000) and Escherichia (t = 4.751, P = 0.000) increased significantly. The relative distribution of the two groups at genus level was shown in Fig. 2(c-d).

At the species level, compared with the normal group, the relative abundance of Lactobacillus Reuteri (t = 7.384, P = 0.000), Lactobacillus Intestinalis (t = 20.252, P = 0.000), Helicobacter Rodentium (t = 8.843, P = 0.000) and Bacteroides Fluxus (t = 17.922, P = 0.000) in the test group were found decreased significantly, that of the Lactobacillus Johnsonii (t = 22.601, P = 0.000), Escherichia Coli (t = 4.755, P = 0.000), Muribaculum Intestinale (t = 8.681, P = 0.000) and Adlercreutzia Equolifacien (t = 15.813, P = 0.000) were found increased. The relative distribution of the two groups at species level was shown in Fig. 2(e-f).

Significance Analysis of Species Differences between the Two Groups

LEfSe difference analysis of multistage species method was used. Each column in the figure represented a kind of species, and the length of the column corresponded to the LDA value. The higher the LDA value was, the greater the difference was. In another words, the absolute value of LDA indicated the influence degree of species with significant differences at different level between the two groups. In Fig. 3, the species differences from class to species level of the two groups were shown from A to E. It could be seen that at different levels, the distributions of Lactobacillus, Enterobacter and Clostridium were significant different between the two groups.

Common Species Analysis between the Two Groups

At the species level, the total number of OTUs (Operational Taxonomic Units, OTU) in the two groups was 1079. There were 642 common OTUs between the two groups. While there were 336 and 101 unique OTUs in the test group and the control group respectively. The total number of OTUs in the test group was higher than that in the control group. The results of this analysis were illustrated in the Venn diagram, shown in Fig. 4a).

Beta Diversity Index Analysis

PCoA analysis was evaluated based on Bray Curtis distance, and the principal coordinate combination with the largest contribution was selected for diagram display. The closer the distance between the points in the diagram, the more similar the species composition structure of the samples was, shown in Fig. 4b).

Analysis of ARGs between the Two Groups

Based on the results obtained from CARD database, the number of reads compared to ARO reference sequences in each group was counted to calculate the relative abundance. The top 20 resistance genes were selected to draw the statistical graph of the relative abundance in each group (Fig. 5a). The cluster heat map of resistance gene (Fig. 5b) was obtained through the abundance clustering. Compared with the control group, the ARGs of ErmF (t = 26.750, P = 0.000) and PmrE (t = 14.068, P = 0.000) in the test group decreased significantly, while resistance genes rpoB2 (t = 19.142, P = 0.000), Tet44 (t = 7.105, P = 0.000), TetW (t = 7.773, P = 0.000) and TetO (t = 6.031, P = 0.000) increased significantly.

Discussion

Outdoor air pollution plays a major role in the increased risk of humans' morbidity and mortality. The fine particulate matter, especially PM2.5, exposed to the environment is a major factor of outdoor air pollution [23]. It has been reported that acute exposure to PM2.5 increased the risk of acute coronary complications, heart failure, venous thromboembolism, arrhythmias and cardiac arrest [24,25]. Likewise, chronic exposure to PM2.5 increased the risk of hypertension, type 2 diabetes and atherosclerosis, as well as the risk of cardiovascular death [26,27]. As mentioned above, these diseases are inextricably related to intestinal flora [13, 14].

In this study, at the phylum level, compared with the control group, the abundance of Firmicutes in the test group decreased, while the abundance of Bacteroidetes increased. Studies have shown that Firmicutes and Bacteroidetes can promote energy absorption and carbohydrate metabolism, produce inflammatory factors and intestinal free radical scavengers, inhibit intestinal inflammation and oxidative stress, and effectively prevent the occurrence and development of gastrointestinal diseases [28, 29]. In previous studies, PM2.5 was thought to affect intestinal flora

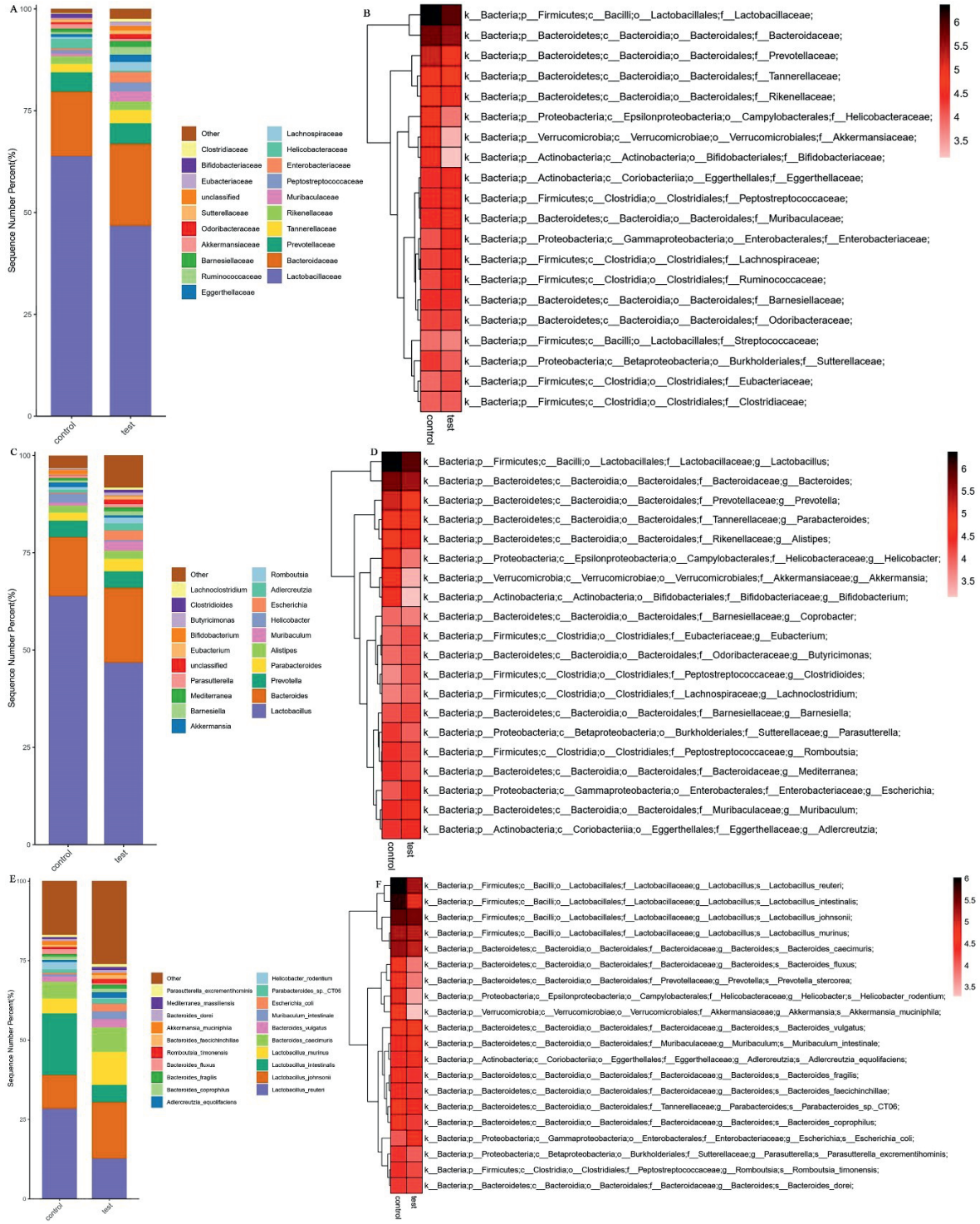

Fig. 2. Relative abundance distribution at family, genus and species level. a) Relative abundance analysis at family level. b) Clustering heat map at family level. c)Relative abundance analysis at genus level. d) Clustering heat map at genus level. e) Relative abundance analysis at species level. f) Clustering heat map at species level.

after passing through the digestive tract [30]. Sze et.al [31] found that exposure to air pollutants affected the associated intestinal bacterial community. It showed that rats exposed to PM2.5 also had the highest amount of intestinal bacterial community content of Bacteroidetes. Another research showed that in haze days, more bacteria belonging to Bacteroidetes, Bacteroidetes and Bacteroidetes were dominant in children who were suffering from asthma [32]. Kish and Salim's study found that intestinal exposure to fine particles caused significant changes in the fecal microbiome in a mouse model of colitis. The abundance of Bacteroidetes and Verrucomicrobia was increased, while the abundance of was Firmicutes decreased in the model group [33,

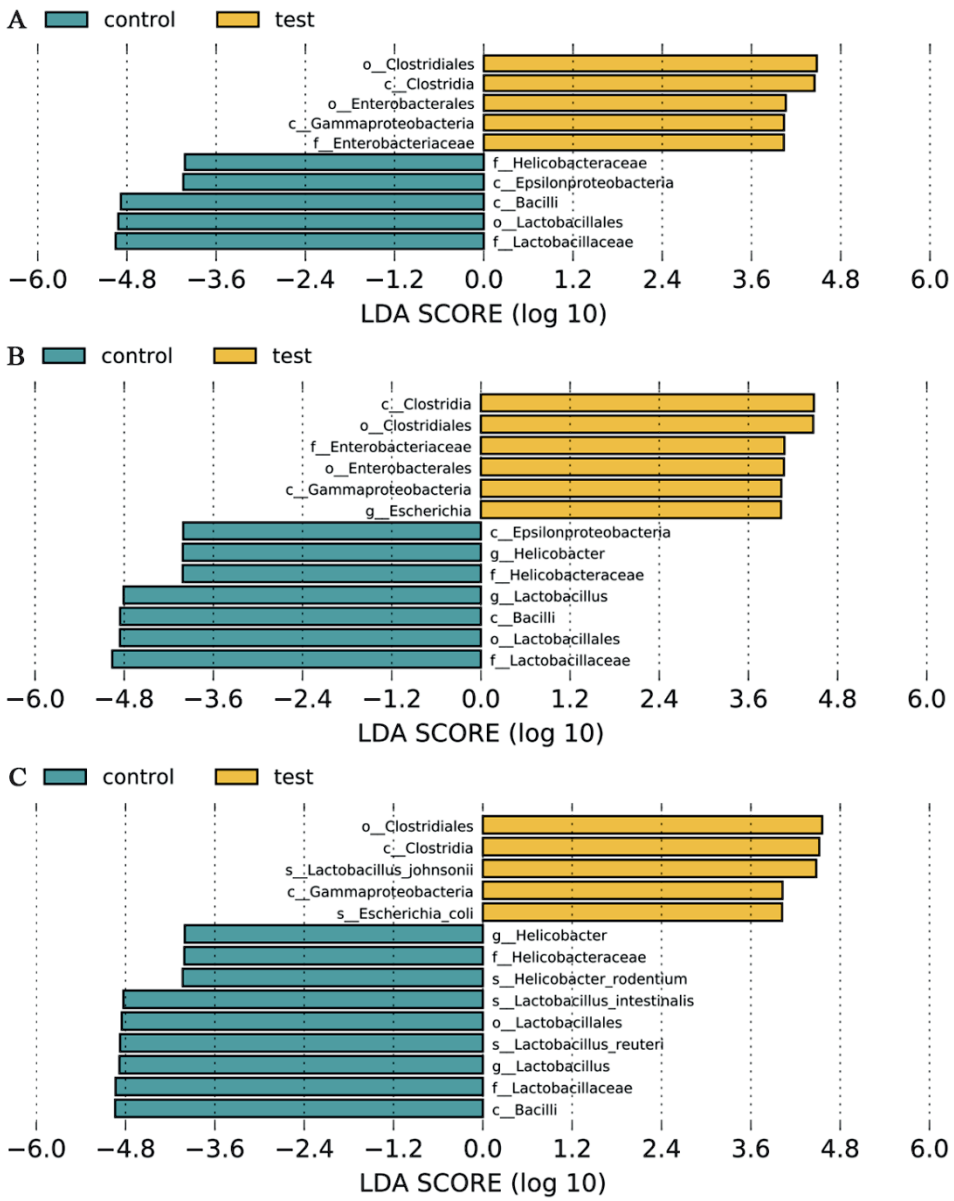

Fig. 3. LDA diagram of LEfSe analysis. a) Family level, b) Genus level, c) Species level.

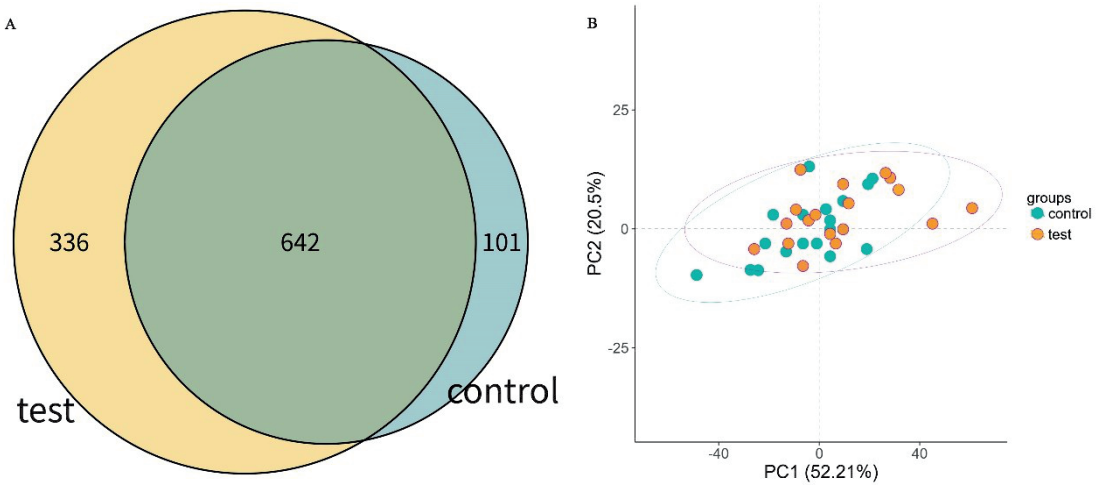

Fig. 4. Sample diversity index analysis. a) Venn diagram of common or unique species. b) PCoA analysis based on Bray Curtis distance.

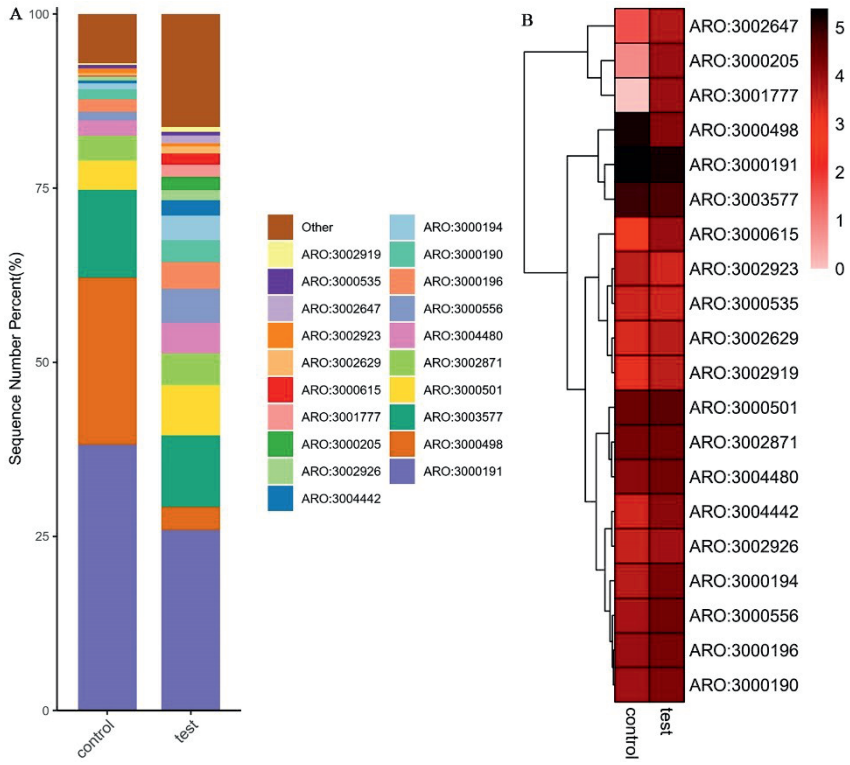

Fig. 5. Analysis of ARGs between the two groups (top 20 genes in relative abundance). a) The relative distribution of ARGs. b) Heat map of ARGs.

34]. At genus level, Muribaculum and Eubacterium were enriched in the test group (Fig. 2c, 2d). At the same time, LEfSe difference analysis showed that Eubacterium and Clostridium were the most different ones in the test group as well (Fig. 3). Many scholars have found that Eubacterium and Clostridium may regulate neural activity in the brain and thus control the body by influencing immune-related pathways related to intestinal flora metabolism [29, 35, 36]. What's more, at the species level, compared with the control group, the relative abundance of Lactobacillus reuteri, Lactobacillus intestinalis and Helicobacter pylori was significantly decreased in the test group, the relative abundance of Lactobacillus johnsoni, Escherichia coli, Muribaculum intestinale and Adlercreutzia Equolifacien was significantly increased. Lactobacillus reuteri is a kind of Gram-positive bacterium, campylobacter with a slightly irregular, rounded tip, which could find in the intestines of all vertebrates and mammals. In recent years, members of the Lactobacillus family have been found to degrade complex polysaccharides to produce short-chain fatty acids such as acetate, butyrate and propionate, which can be used as energy by the host [37]. Among them, Lactobacillus reuteri DSM 17938 (LR), belonging to the Genus Lactobacillus, is an effective regulator of the immune system and the "shaping agent" of intestinal flora, as well as the regulator of plasma and fecal metabolomics characteristics, each of which may contribute to the improvement of early intestinal inflammation [38]. The decrease of this sorts of flora in the test group may also be related to the decrease of immune system function and the production of ARGs.

Sequence alignment and annotation results of CARD database showed that most of ARGs, such as rpoB2, Tet44, TetW and TetO increased in test group compared with the control group. According to CARD database, the rpoB2 belongs to rifamycin antibiotic. The Tet44, TetW and TetO belong to tetracycline antibiotics. Tet44 is a kind of tetracycline resistance gene found in Campylobacter fetus, and binds to the ribosome to confer antibiotic resistance as a ribosomal protection protein. TetW is a sort of ribosomal protection protein. It is associated with both conjugative and non-conjugative DNA and has been found in strains of C. difficile. TetO is a ribosomal protection protein as well. It is associated with conjugative plasmids. Some researchers have shown that ARGs can be transmitted through polluted atmospheric particles [39]. Cáliz et al. [40] have also shown that ARGs sul1(resistance to sulfonamides), TetO (resistance to tetracycline) and intI1(a proxy for horizontal gene transfer and anthropogenic pollution) can be dispersed in tropospheric aerosols for long and sustained periods. They also revealed that the intercontinental diffusion of ARGs and the global spread of antibiotic resistance had the potential predictability of interannual variability and remote deposition rates. The decrease of normal dominant flora reflected to the increase of opportunistic pathogens, which was related to the carried ARGs [30]. Some scholars [41] found that systemic lupus

erythematosus was positively correlated with exposure to several kinds of atmospheric pollutants. Particulate matter had an impact on autoimmune disease in areas with a high incidence of newly diagnosed systemic lupus erythematosus [42]. Gilcrease et al. [43] suggested that pollution, especially particulate matter, might be one of the important triggers of inflammation and autoimmunity. All of these factors may be responsible for the increased proportion of resistance genes in the intestinal flora after PM2.5 inhaled.

There were some limitations in this study, including a relatively small sample quantity and limited regional sources of PM2.5. Also, the intestinal microbial system is very complex and susceptible to a variety of factors, which are difficult to control in free-living individuals with inherent genetic variation [44]. Nevertheless, a balanced gut microecosystem is one of the most important factors to human health. Some health-related problems may be addressed by the balanced microecosystem [45]. It is of great significance to study the interaction mechanism between PM2.5 inhaled and intestinal flora for the prevention of social diseases.

Conclusion

It could be seen on the analysis from phylum level to species level, the abundance of bacteria in the test group changed significantly compared with that in the control group. At the phylum level, the Firmicutes in PM2.5 inhaled rats decreased and the Bacteroides increased. PM2.5 exposure can change the expression of ARGs in intestinal flora. The increasing proportion of ARGs in the test group suggested an indirect manifestation of imbalance of intestinal flora. Controlling air pollution and reducing PM2.5 emissions are crucial to maintaining public health, especially in developing countries.

Authors' Contribution

Ben Liu, Xueying Cheng, Yongzhi Lun designed the experiments. Ben Liu, Xueying Cheng, Fanghua Zhang, Wen Dong and Qi Lin performed the experiments. Ben Liu, Yongzhi Lun and Fanghua Zhang analyzed the data. Ben Liu and Xueying Cheng wrote the manuscript.

Conflict of Interests

There is no potential conflict of interest to declare.

Ethical Approval

This study was approved by the Ethics Committee of Putian University, China.

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Informed Consent

All the study subjects were informed of the significance of this study and signed informed consent forms.

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