Exposure to atmospheric pollutants is associated with alterations of gut microbiota in spontaneously hypertensive rats

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Abstract. Atmospheric particulate matter with a diameter <2.5 μm (PM2.5) and pollution are worldwide environmental problems and may have negative effects on cardiovascular disease through the lung and gut. The dynamics of intestinal microflora in response to particulate pollutants is unclear. The present study investigated changes in the gut microbiota related to pollutant exposure using spontaneously hypertensive rats (SHR). DNA was extracted from fecal samples. Amplicon Generation and the quality control of PCR products were performed. PCR products was sequenced on an Illumina HiSeq 2500 platform. Data analysis included: operational taxonomic unit (OTU) clustering and species annotation, alpha diversity, beta diversity, principal coordinates analysis (PCoA), and the use of PICRUSt bioinformatics software. The microbial diversity of the SHR rats was inversely associated with exposure to pollutants. In terms of relative abundance, 24 bacterial genera and 2 genera in particular (*Actinobacillus* and *Fusobacterium*) significantly declined, and one genus (*Treponema*) increased. Moreover, pollutant exposure was associated with the accumulation of genes from the gut microbiota that are implicated in cardiovascular diseases. From the long-term exposure experiment, rats appeared to respond to pollutant injury. In conclusion, these results suggest that the effects of atmospheric pollutants on organisms are not limited to the respiratory tract, but also include the gastrointestinal tract. Pollutants are likely to influence the intestinal microbiota and promote the progression of cardiovascular disease.

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Introduction

The effect of air pollution on the human body has become an important problem in China and the focus of research. According to epidemiological data, mortality due to respiratory disease in north China significantly correlates with winter fog and haze (1). Mixed pollutants, including particulate matter (PM) and sulfide and nitrogen oxides, have become the main causes of cardiovascular diseases in winter (2). The deadliest form of air pollution during haze is atmospheric particulate matter with a diameter <2.5 μm (PM2.5) (3). PM2.5 is associated with increased morbidity and mortality from respiratory and cardiovascular diseases such as cardiac arrhythmia, congestive heart failure, and ischemic heart disease (4,5).

Although most research on the links between pathology and pollution have focused on cardiovascular and respiratory effects, there are also indications that air pollutants affect the human gastrointestinal tract (6). Epidemiological studies have revealed associations between air pollution exposure and various gastrointestinal diseases, including inflammatory bowel disease (7), appendicitis (8), irritable bowel syndrome (9) and enteric infections in infants (10). A rise in total measured air pollutants was associated with an increase in hospitalizations for inflammatory bowel disease (11) and abdominal pain (3). Airborne PM2.5 in particular increases the permeability of the intestinal gut, disrupting the epithelial barrier and thereby triggering gastrointestinal disorders (11).

The gut microbiota is an important contributor to human health (12). A dysfunctional gut microbiome has been implicated in the development of many disorders, such as diabetes (13), obesity (14) and cardiovascular disease (15). A study of pollutants reported that smoking may exert pathological effects, at least in part by regulating intestinal microbiota (11).

Little is known about how air pollution influences the gut microbiome (16), but there is evidence that the gastrointestinal tract can be affected by respiratory tract exposure to PM2.5 (17). Human studies have shown that mucociliary air pollutants are cleared quickly from the lungs (18). The documented effect of air pollutants on the respiratory and gastrointestinal systems, and the effect of tobacco smoke on intestinal microbiota, supports the need for more information regarding how PM2.5 may influence the gut microbiota.

Spontaneously hypertensive rats (SHR) have been deemed suitable for studying interactions of the digestive and respiratory systems under air pollutant exposure (11). To gain greater understanding of such interactions, in the present study we investigated alterations in the intestinal flora microbiota of rats challenged by PM2.5 exposure, using 16S rDNA sequencing.

Materials and methods

SHR rats. Specific pathogen-free SHR male rats (n=10; aged 8-11 weeks; weight 200±10 g) were purchased from Beijing Weitong Lihua Animal Technology (license number SCXK Beijing 2012-0001) and housed at 22±2˚C and 45-55% humidity, with natural day and night hours and natural light. Food and water were provided *ad libitum*. Adaptive feeding was allowed for one week prior to experiments while their activity and eating was observed.

The present study was approved by the Ethics Committee of the School of Basic Medical Sciences, Jilin University (Changchun, China).

Source and treatment with PM2.5. The PM2.5 used in the present study was collected from Shenyang city atmosphere with a 120 F flow dust sampler (sampling flow, 1,000 l/min). A suspension of PM2.5 dust (4 mg/ml) was prepared with sterile saline solution (19).

Preparation of mixed gases. Standard mixed gas was provided by Dalian Special Gas Industry (SO_2 2013.1x10^{-6}; NO_2 1187.0x10^{-6}; CO 23199.6x10^{-6}). The cylinder filling pressure was 9.0 MPa and the volume was 40 l.

Animal handling. The Institutional Animal Care and Use Committee of Shenyang Medical College approved the protocols for handling the rats. The rats were anesthetized with an inhalation of nose and mouth of chloral hydrate (0.7 mg/100 g) prior to each treatment with PM2.5 dust-saline.

The rats were dosed with PM2.5 dust-saline (1 ml of 4 mg/ml) once per week for 12 weeks, using a non-exposed tracheal perfusion method (19). Each rat was treated individually to ensure the dosage. The rats breathed mixed gases (SO_2 2013.1 mg/m^3; NO_2, 1187.0 mg/m^3; CO, 23199.6 mg/m^3) by dynamic inhalation (19). The flow rate of the mixed gas was 1.2 l/min, each exposure time was 3 h.

The control group rats inhaled normal air in the cage. During the experimental period, each rat was housed individually in separate cages.

Sampling set up. Fecal samples were collected randomly from 3 rats at 0, 7, 15, 30, 60 and 90 days, <5 g fecal sample was collected from each individual.

Stool DNA extraction. A Qiagen 51504 QIAamp DNA Stool Mini kit (Qiagen GmbH) was used to extract the total DNA. The quantity of the extracted DNA was determined with a NanoDrop 2000 (Thermo Fisher Scientific, Inc.).

Amplicon generation and the quality control of PCR products. The PCR primers were selected based on the sequences of the V3 and V4 hyper-variable regions of the bacterial 16S rRNA gene. The microbial V3-V4 region was amplified by PCR using the primers F: 5'-ACTCCTACGGGAGGCAGCA and R: 3'-GGACTACHVGGGTWTCTAAT (20). All PCR reactions were performed with Phusion High-Fidelity PCR Master Mix (New England Biolabs, Inc.). The quality of the PCR products was determined by electrophoresis with 2% agarose gel for detection. Samples with bright main strips between 400-450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. The mixed PCR products were purified with a Qiagen Gel Extraction kit (Qiagen).

Library preparation and sequencing process. Sequencing libraries were generated using a TruSeq DNA PCR-free sample preparation kit (Illumina, Inc.) in accordance with the manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit 2.0 fluorometer (Thermo Fisher Scientific, Inc.). Lastly, the library was sequenced on an Illumina HiSeq 2500 platform (Illumina, Inc.) and 250-bp paired-end reads were generated.

Data analysis. The reads were merged using the FLASH tool, which merges paired-end reads from the original DNA fragments. Quality filtering on the raw tags was performed under specific filtering conditions in accordance with the QIIME (v1.9.1) quality-controlled process. Sequences that overlapped by more than 10 bp were assembled and junk reads were discarded.

Production sequences with ≥97% similarity were assigned to the same operational taxonomic unit (OTU). The representative sequence for each OTU was screened for further annotation. Species annotation for each representative sequence was picked from each OTU. The Green Gene Database was used based on the RDP3 classifier phylogenetic tree. The relatedness of different OTUs, and the differences in the dominant species in different samples (groups), was conducted using MUSCLE software (version 3.8.31). OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences.

Alpha diversity and the community richness index were calculated with QIIME (version 1.9.1) and displayed with R software (version 2.15.3). Beta diversity of weighted and unweighted UniFrac values were calculated using QIIME (version 1.9.1). Cluster analysis was preceded by principal component analysis with the 'ggplot-2' package in the R software (version 2.15.3). Principal coordinates analysis (PCoA) was performed to obtain the principal coordinates and visualize the complex multidimensional data. UPGMA (unweighted pair group method with arithmetic mean) clustering was performed for hierarchical clustering to interpret the distance matrix using average linkage and was conducted using QIIME (version 1.9.1).

PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) bioinformatics software was applied for predicting the gene family abundance of bacterial communities, based on the 16S rDNA gene data and a database of reference genomes (21). PICRUSt consisted of two steps, gene content inference and metagenome inference, performed as previously described (22). The t-test (SPSS 19.0;

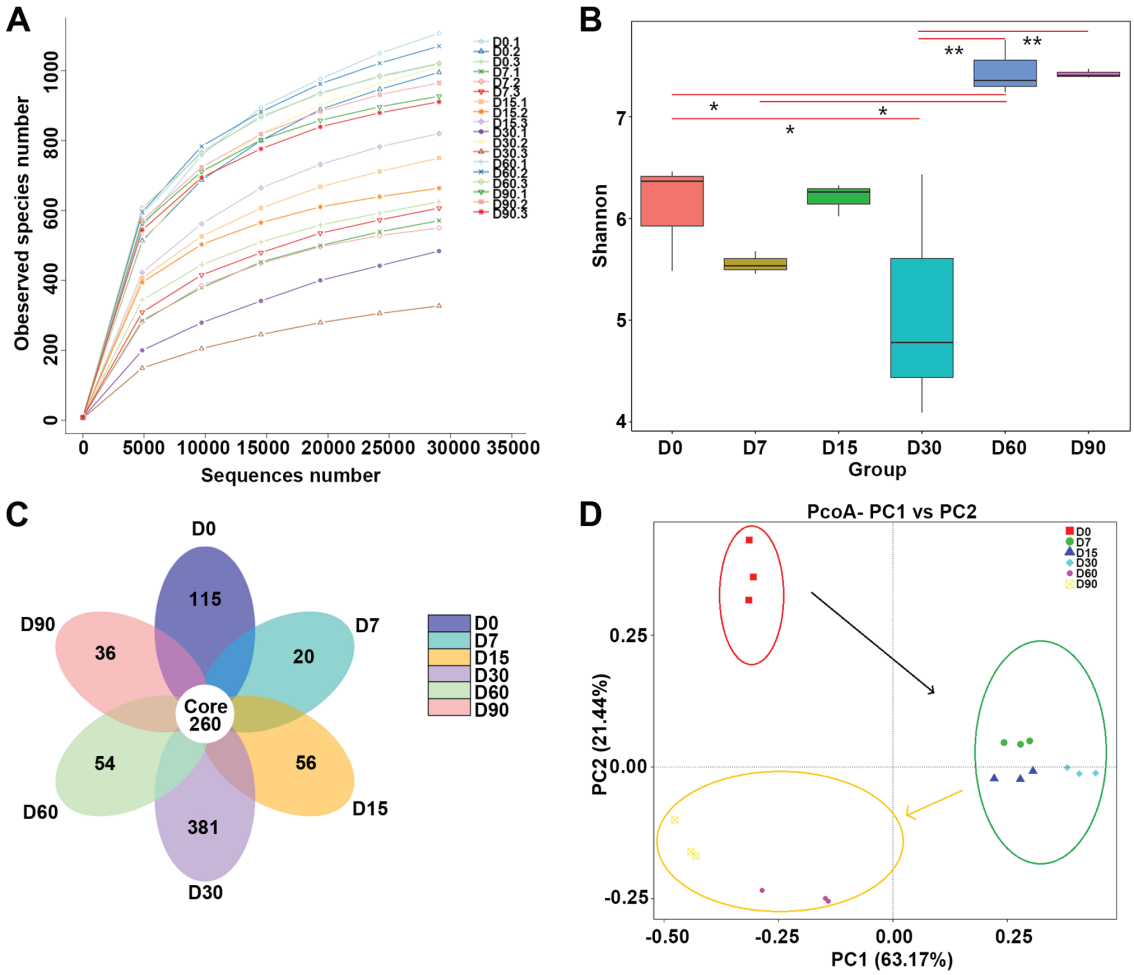

Figure 1. Different microbial diversity indices in different groups. (A) Bacterial rarefaction curves based on observed species were used to assess the depth of coverage for each sample. (B) Box-plot of grouped Shannon index, showed the variation trend of diversity. *P<0.05, **P<0.01. (C) Flora data showed the number of shared OTUs and the number of independent OTUs, shared OTUs was shown in the core area, and the shared OTUs were in the petal. (D) Weighted Unifrac PCoA showed similarity among the bacterial communities associated with each sample.

Chicago, IL, USA) was used to analyse the data with normal distribution. P<0.05 was defined as the standard criterion for statistical significance (23).

Results

Changes in the gut microbiota associated with PM2.5 exposure. To investigate changes in the gut microbiota in response to PM2.5, we performed amplicon sequencing of the fecal samples from the exposed rats at 6 sampling timepoints (0, 7, 15, 30, 60 and 90 days). Rarefaction curves revealed no new observed species after 20,000 reads, which meant that almost all bacterial species were detected in all samples (Fig. 1A).

Based on 97% sequence similarity, all the sequences of regions V3-V4 were clustered into 10,887 bacterial OTUs. The diversity of the microbial communities was measured using Shannon diversity indices (Fig. 1B). We found that pollutant treatment was associated with these results; there was a significant reduction in bacterial diversity at 7 days. At day 60, the bacterial diversity was higher (Shannon index = 7.45) than at day 0 (Shannon index = 6.103). According to the flora data (Fig. 1C), the fecal samples shared 260 different OTUs. The day-30 fecal sample was the most populous among the independent OTUs (381 independent OTUs), and the next populous was the day 0 sample (115 independent OTUs).

The beta diversity of the bacterial communities associated with rats was investigated through PCoA, which was performed on the phylogenetic beta-diversity matrix obtained by UniFrac (Fig. 1D). The samples exhibited good repeatability. Moreover, ANOSIM of the weighted UniFrac distances revealed significant differences in the bacterial communities between groups (R=0.0689, P=0.033).

Intestinal bacteria at the phylum level. Taxonomic assignment analysis at the phylum level is shown in Fig. 2A. After the bacterial OTU representative sequences were taxonomically classified, the results showed that the most abundant and common phyla (abundance within the community ≥1%) in all samples were *Firmicutes* (35%), *Bacteroidetes* (29%), *Proteobacteria* (17%) and *Tenericutes* (1%).

Over time, the relative abundance of bacteria changed to a great extent at the phylum level (Fig. 2B-E). Statistically significant differences of the top phyla were found (compared with the day 0 sample), as follows: day 7, *Firmicutes*, *Bacteroidetes* and *Tenericutes*; day 15, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Tenericutes*; day 30, *Bacteroidetes*; day 60, *Proteobacteria*

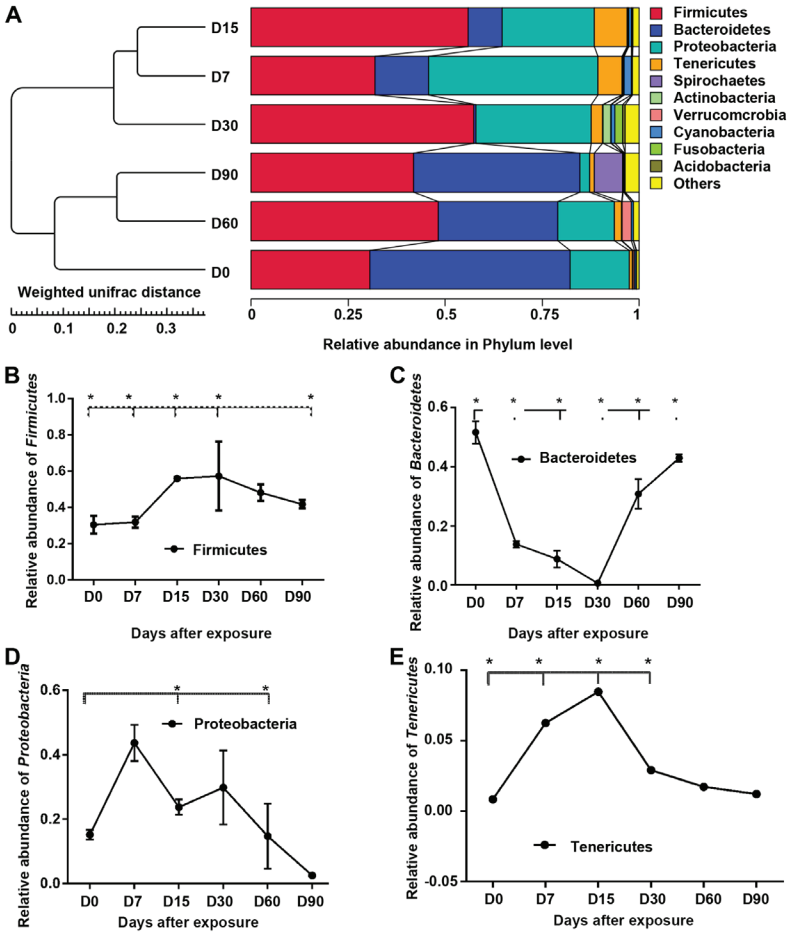

Figure 2. Main components of intestinal bacteria at the phylum level. (A) Relative abundance of the RDP-classified sequence reads at the phylum level (TOP 10). (B) Shifts and the analysis of significance in the relative abundance of *Firmicutes* with all samples $^{*}P<0.05$. (C) Shifts and the significant analysis of relative abundance of *Bacteroidetes* with all samples $^{*}P<0.05$. (D) Shifts and the significant analysis of relative abundance of *Proteobacteria* with all samples $^{*}P<0.05$. (E) The shifts and the significant analysis of relative abundance of *Tenericutes* with all samples $^{*}P<0.05$.

and *Tenericutes*; and day 90, *Firmicutes* and *Bacteroidetes*. Compared with the day 0 sample, after 30 days of exposure *Firmicutes* increased by 37% and *Tenericutes* increased by 44%, and there were significant decreases in *Bacteroidetes* (-17%) and *Proteobacteria* (-83%).

Alterations in the gut bacterial compositions. To investigate further the effects of air pollutants on the intestinal microflora of SHR rats, 209 genera were identified from the gut bacterial communities of the samples. Among these, 9 abundant genera constituted >0.1% of the total sequences in at least one sample (Fig. 3A). These were: *Prevotellaceae-Prevotella* (37.25%), *Bacillus* (0.45%), *Veillonella* (1.23%), *Achromobacter* (3.11%), *Helicobacter* (2.31%), *Lactobacillus* (1.42%), *Campylobacter* (0.59%), *Paraprevotellaceae-Prevotella* (6.93%) and *Oscillospir*a (2.41%).

Compared with the day-0 sample, the most significant differences in OTUs at the genus level were the following (Fig. 3B and C): *Cetobacterium*, *Mycoplasma*, *Treponema*, *Actinobacillus*, *Prevotella*, *Odoribacter*, *Achromobacter*, *Spironema*, *Fusobacterium*, *Campylobacter*, *Clostridium* and *Parvimonas*. The result showed that two genera (*Actinobacillus* and *Fusobacterium*) significantly decreased after 30 days. However, the bacterial community showed significant increases in relative abundance of only one genus (*Treponema*). During 30 days of incubation, other bacterial genera showed dramatic fluctuations in abundance but, no changes were observed after 30 days.

Functional maturation of the gut bacterial community and the shifts of disease-involved genes in gut metagenomics. To investigate how pollutants affect the functional profile of gut microbiota, PICRUSt analysis was used to analyse the KEGG pathway compositions in bacterial populations (Fig. 4A). The result suggested that most predicted genes were enriched in the following pathways: organismal systems, cellular processes, environmental information processing, human diseases, genetic information processing and metabolism.

The changes in most genes from treatment groups of SHR rats did not correlate with the time-points (Fig. 4B). Changes in gut function or genes in the SHR rats were not linear after prolonged exposure to the pollutants. Genes related to human diseases increased significantly (23%) prior to 30 days (Fig. 4C). Surprisingly, the genes related to human diseases significantly decreased after long-term pollutant exposure (15% lower at day 90 compared with that of day 0).

The percentage of genes linked to cardiovascular diseases peaked at day 15 (Fig. 4D). Moreover, the genes detected before

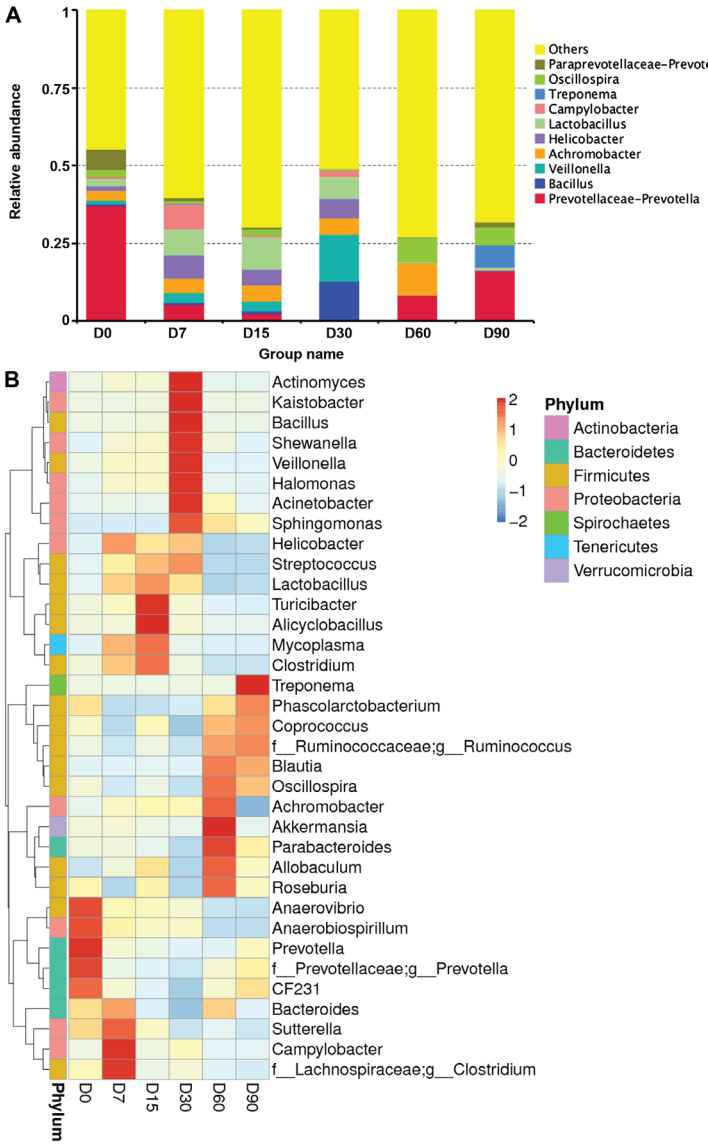

Figure 3. Significant alterations in the gut bacterial compositions. (A) Relative abundance and the average proportional distributions of gut bacterial Top 10 genus identified in all groups. (B) Heat map and hierarchical clustering of genera in the gut bacterial communities of all samples. The color of the squares on the left indicate the average abundance of the OTU in each group. The OTUs are ordered via phylogenetic positions.

day 15 were all significantly higher than the baseline (day 0), and then decreased after 15 days. At day 90, the percentage of genes related to cardiovascular disease was significantly lower than that at day 0.

Most genes were annotated and enriched to the environmental information-processing pathway (Fig. 4E). Compared with that of day 0, the enrichment of related genes increased significantly at day 7, 15, 30, 60 and 90 (Fig. 4E).

Discussion

In the present study, we investigated shifts in gut microbial populations in SHR rats within 90 days of continuous exposure to PM2.5 air pollutant. The results revealed changes in gut microbiota composition and functional adaptation of the gut bacterial community associated with long-term pollutant exposure.

A recent study showed that *Bacteroidaceae*, *Oscillospira*, and *Ruminococcus* are the most abundant genera in the guts of Wistar rats reared under normal conditions (24). By contrast, the present study found that *Prevotellaceae-Prevotella*, *Paraprevotellaceae-Prevotella* and *Achromobacter* were the most abundant. The gut microbiota structure of the SHR rat is different from those of Wistar rats (1). In previous studies, PM2.5 was thought to affect the microbial community in the gut after going through the digestive tract (25). In this study, we found a significant decrease in alpha diversity in the gut bacterial community associated with exposure to air pollutants. To some extent, damaging effects of air pollutants on gut microbial diversity were found in animal experiments.

In the present study, after long-term exposure the restoration of intestinal microbial diversity indicated that the intestinal system of rats has a certain ability to maintain microbial diversity. Whether humans have such rapid recovery is unknown. The decrease in diversity during the early days (0-15 days) of exposure indicated gut diseases and simplification of metabolic types. This matches previous epidemiological investigation results, in which PM could

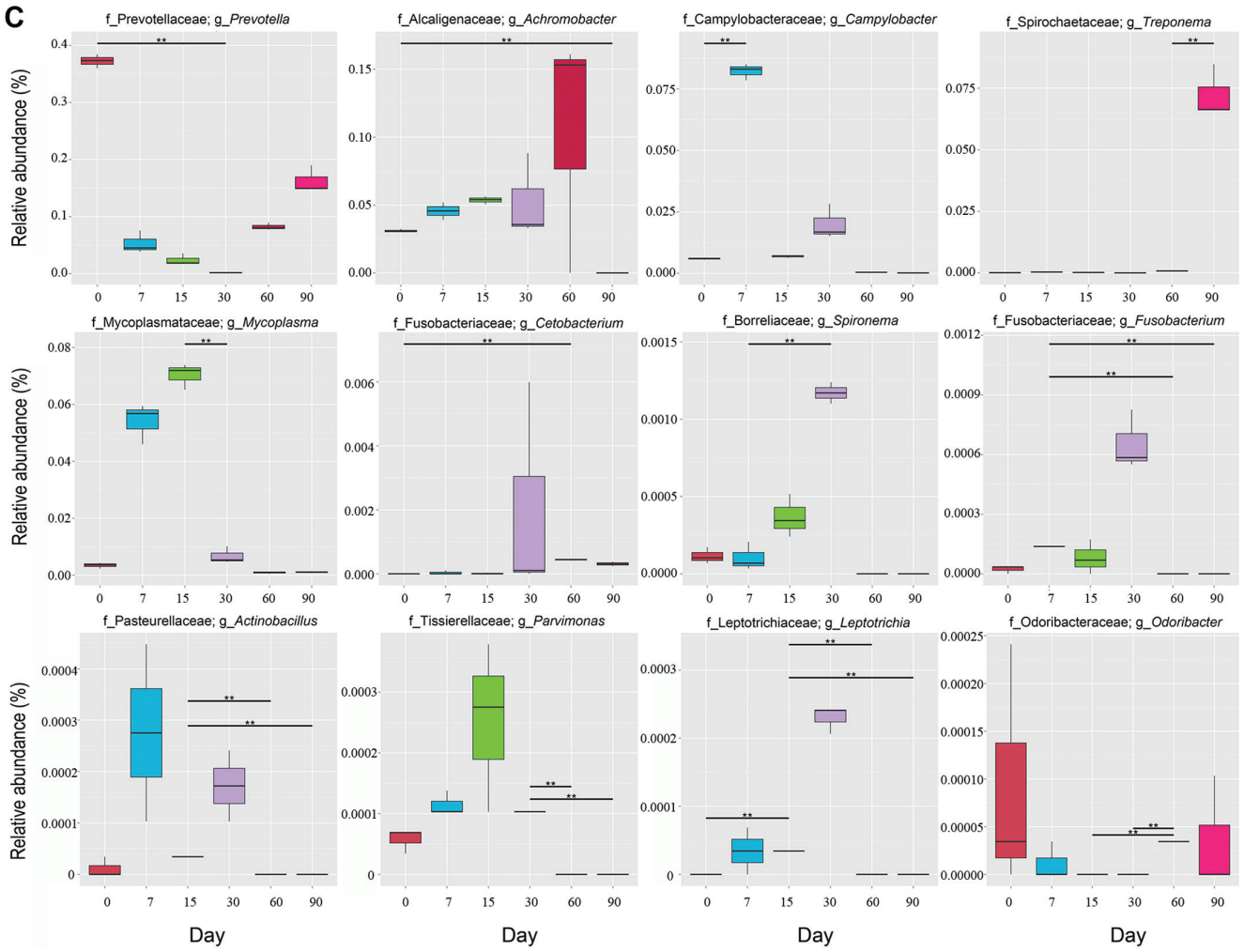

Figure 3. Continued. (C) MetaStat analysis showing the most differential OTUs (genus level) from all samples. Extreme significant difference that was obtained between the samples is indicated; (**P<0.01).

alter the gut microbiome and result in gut disease (6). How this process was triggered and whether it is related to the influence of PM2.5 on microbiota colonization in the gastrointestinal tract (26) remains unknown. In particular, in the present study the change in gut microbes in the SHR rats was characterized by a major transition from *Bacteroidetes* to *Firmicutes* under pollutant exposure (Fig. 2A). Our results showed significant decline in the relative abundances of 2 phyla (*Bacteroidetes* and *Spirochaetea*) after 90 days of long-term exposure, and the number of phyla did not significantly increase. In the present study, pollutant exposure did not affect the structure of the phyla in the SHR rats, but the quantitative changes were significant and responded rapidly to pollutant exposure.

At the genus level, *Cetobacterium*, *Mycoplasma*, *Treponema*, *Actinobacillus*, *Prevotella* and *Odoribacter* were the genera that declined most significantly after 90 days (>5% decline). Mycoplasma and Treponema are specific pathogens that are associated with chronic respiratory infections (28). These pathogens that are common in the respiratory tract, which changed dramatically in the intestinal tract, and the mechanism of this change is unclear. Moreover, the flora of the lungs and intestines may interchange and colonize via the lymphatic system (25). Our experiments seem to confirm this finding.

Prevotella is a newly discovered strain in a recently isolated genus that includes 20 species (16), with the most common species being a black pigmented strain (*P. melaninogenica*). This genus is mainly concentrated in the healthy human oral cavity, female genital tract (29). It is a common opportunistic pathogen in the clinic and can cause endogenous infections, especially of the female genital tract and oral cavity (29). Thus, *Actinobacillus* may be a candidate pathogen of the host upper respiratory tract, digestive tract, and urogenital tract; it belongs to thousands of normal flora. In the present study, the change in the abundance of *Prevotella* may be related to the oral perfusion of the pollutants.

Odoribacter was reported as enriched in mice with colorectal cancer and may be related to tumor development (30). Genera of the bacterial phyla *Cetobacterium* may be related to digestive tract function. *Cetobacterium* is associated with the biosynthesis of acetic acid (31).

It is reported that intestinal microbiota metabolism of choline/phosphatidylcholine produces trimethylamine (TMA), which is further metabolized to a proatherogenic species, trimethylamine-*N*-oxide (TMAO) (15). TMAO is

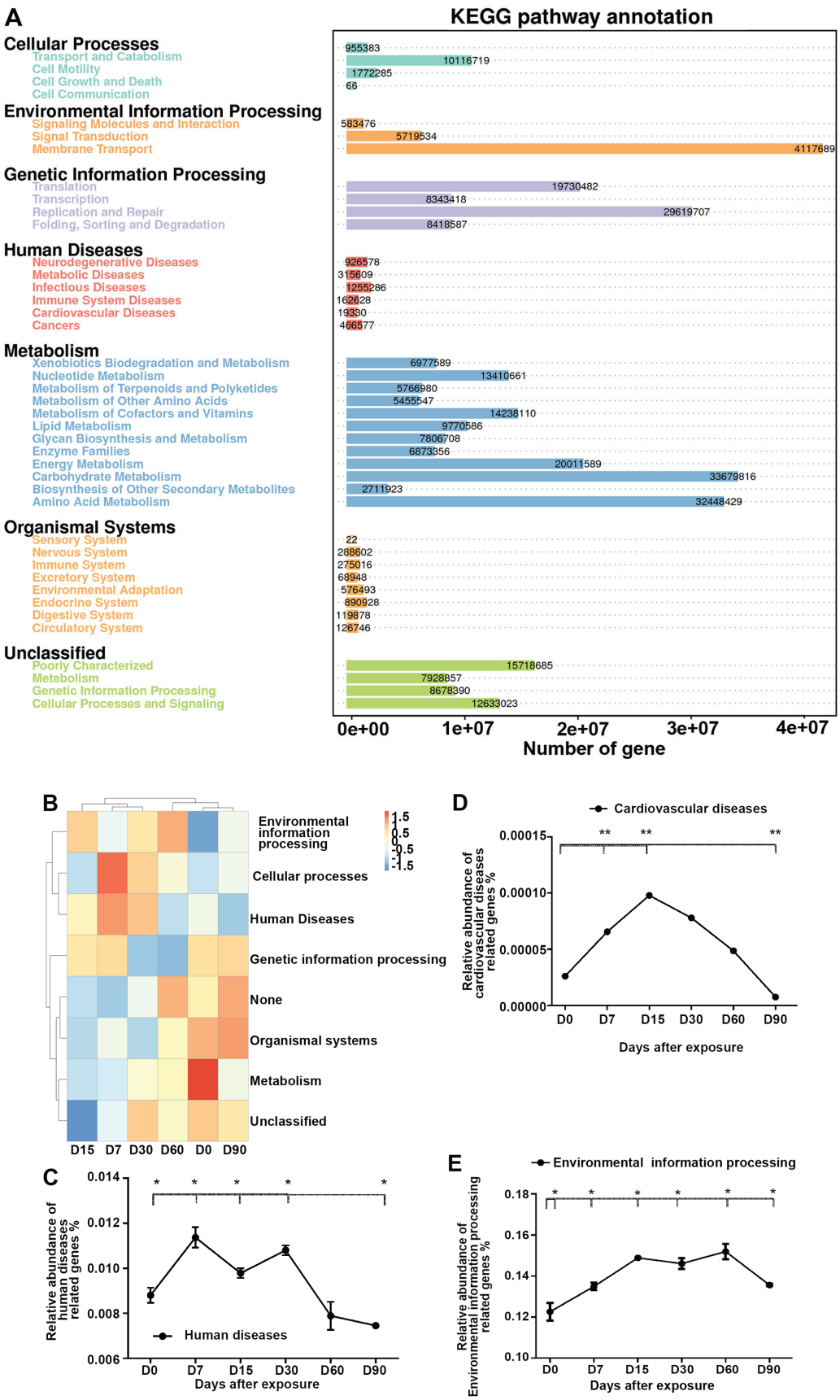

Figure 4. KEGG pathway annotation and the quantitative distribution of the gene enrichment. (A) Overview of the predicted data. (B) Shifts in gut bacterial functional profiles as the pollutant treated SHR rats. Heat map and hierarchical clustering of differentially abundant KEGG pathways identified at 6 sampled time-points (0, 7, 15, 30, 60 and 90 days). The values of color in the heat map represent the normalized relative abundance of KEGG pathways (log 10). Heat map and hierarchical clustering of differentially abundant KEGG pathways identified at 6 sampled time-points (0, 7, 15, 30, 60 and 90 days). The values of color in the heat map represent the normalized relative abundance of KEGG path. (C-E) Analysis was performed to identify the significantly differentially abundant of selected pathways (human diseases, cardiovascular diseases, and environmental information processing) among groups and day 0 sample. Asterisks indicate the significant differences that were obtained between D0 sample and samples of following observational days ($^{*}0.01<P<0.05$, $^{**}P<0.01$).

closely related to the increased occurrence of major adverse events of cardiovascular diseases. The bacterial community and its composition provide different TMAOs (32). Increased TMAO levels will significantly affect systemic cholesterol accumulation, leading to increased generation of atherosclerotic plaque (33).

The gut microbiota have also been proven to be involved in the anabolism of TMAO as the producer of the precursor (TMA) (34). Microecology studies have shown that higher TMAO plasma concentrations were associated with the *Prevotella* enterotype, as opposed to the *Bacteroides* enterotype (34). In the present study, from investigation at the genera level we found that *Prevotellaceae-Prevotella* (relative abundance 37.25%) is the most enriched genus in all samples. Moreover, pollutant exposure reduced the abundance of this population within 30 days (Fig. 3C). This may be related to the intestinal microbial background of SHR rats (24), the relative abundance of Bacteroides was decreased after treatment with pollutant (Fig. 3A). This may suggest that a metabolic disorder of TMAO leads to an increased risk of cardiovascular disease at the microbial level.

PICRUSt is a closed-access analysis based on an established database. It is not comprehensive, but can provide accurate directional guidance. In this study, the rat intestinal metagenome responded to pollutant exposure within 30 days (Fig. 4). After 30 days, the intestinal metagenome showed an ability to repair damage. The accumulation of cardiovascular disease-related genes in the gut showed a statistically significant association with air pollutants. This is consistent with other reports (33). Previous studies have shown that, in response to changes in environmental factors, the changes in microbial functional diversity were greater than changes in system diversity (35). The evidence from metagenomic studies may be more useful for clinical studies than that of microbial systematic investigations.

In conclusion, herein the effects of air pollutants on the gut microbiota of SHR rats are reported. Moreover, statistically significant changes in the microbiota were investigated. Changes in phylum levels indicate that the intake of air pollutants highly affects the lower taxa, and abnormalities in metabolism and nutrient absorption may be triggered by the intake of air contaminants. The pathological features of these changes require further investigation. The results of this study support that the consequences and destruction of air pollutants on the microbial structure of the intestinal tract are no less than that of the respiratory system.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DC wrote the manuscript. CX and HJ helped with the animal handling. DC, BY and JN contributed to DNA extraction and PCR. SY and YS were responsible for the data collection and analysis. YZ and XW were in charge of the library preparation and sequencing process. The final version was read and adopted by all the authors.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the School of Basic Medical Sciences, Jilin University (Changchun, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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