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Original article

Bacterial communities in PM_{2.5} and PM₁₀ in broiler houses at different broiler growth stages in spring

J. Zhang¹, Y. Li², E. Xu², L. Jiang¹, J. Tang¹, M. Li¹, X. Zhao¹, G. Chen¹,
H. Zhu¹, X. Yu¹, X. Zhang¹

¹ College of Life Science, Ludong University,
No. 186 Hongqi Middle Rd, Zhifu District, Yantai 264025, Shandong, China

² Shandong Veterinary Drug Quality Inspection Institute,
No. 68 Huaicun Street, Huaiyin District, Ji'nan 250022, Shandong, China

Abstract

The welfare and healthy growth of poultry under intensive feeding conditions are closely related to their living environment. In spring, the air quality considerably decreases due to reduced ventilation and aeration in cage systems, which influences the meat quality and health of broilers during normal growth stages. In this study, we analyzed the airborne bacterial communities in PM_{2.5} and PM₁₀ in cage broiler houses at different broiler growth stages under intensive rearing conditions based on the high-throughput 16S rDNA sequencing technique. Our results revealed that PM_{2.5}, PM₁₀ and airborne microbes gradually increased during the broiler growth cycle in poultry houses. Some potential or opportunistic pathogens, including *Acinetobacter*, *Pseudomonas*, *Enterococcus*, *Microbacterium*, etc., were found in the broiler houses at different growth stages. Our study evaluated variations in the microbial communities in PM_{2.5} and PM₁₀ and potential opportunistic pathogens during the growth cycle of broilers in poultry houses in the spring. Our findings may provide a basis for developing technologies for air quality control in caged poultry houses.

Key words: bacterial communities, broilers, high-throughput sequencing, particulate matter

Introduction

Airborne particulate matter (PM) is a mixture of airborne particles originating from the breakdown of crustal components or human activities. The size of ambient particles is directly related to their capacity to cause health concerns. Fine particles less than 2.5 μm in diameter (PM_{2.5}) can enter the pulmonary alveoli

as well as the deep part of the respiratory tract (alveolar and bronchia) or even pass into the blood stream through the blood-gas barrier and thus have more adverse effects on humans and animals (Daniels et al. 2000, Hsieh et al. 2008, Franck et al. 2011). Inhalable coarse particles between 10 and 2.5 μm in diameter (PM₁₀) can enter the respiratory tract through the nasal cavity and throat and then accumulate in the respiratory

system and induce various respiratory diseases (Menichini et al. 2001, Sharma et al. 2007). PM contains complex aggregates of inorganic materials, smoke, metal elements, all types of liquid and solid materials, and microbial aerosols, such as bacteria, fungi, and viruses (Schlesinger and Cassee 2003, Grahame and Schlesinger 2005).

Intensive livestock and poultry farming releases high levels of microbial aerosols (Crowe et al. 1996, Cambra-Lopez et al. 2010, Lawniczek-Walczyk et al. 2013). Microbial analyses of poultry houses have revealed that microbial aerosols play crucial roles in the air population and affect the healthy growth and production performance of birds (Hong et al. 2012, Just et al. 2012). Birds are a rich reservoir of microbes and an important source of biological aerosols. In broiler houses, the composition and concentration of biological aerosols were correlated with the microflora present in and on broilers, which to a certain extent reflected the hygiene and health of the broilers as well as recessive infections, the pathogen carrying status and their diseases (Lovanh et al. 2016). The construction of broiler houses has ensured the maintenance of a stable indoor environment that is represented by a constant temperature, relative humidity, flow speed and lighting. However, the difficulty in controlling the indoor environment increases with broiler growth. Poor air fluidity and higher humidity create a 'hotbed' for microorganism survival and reproduction. Thus, the atmosphere in poultry houses contains microbial aerosols that are characterized by complex structures and high microbial diversity (Dungan 2010). These aerosols not only affect the growth and development of the birds but may also cause diseases through the spread of pathogenic agents resulting from air exchange (Heederik et al. 1991). Many pathogenic bacteria, opportunistic pathogens and nonpathogenic microbes are contained in these aerosols, which have been reported to be closely related to the welfare and healthy growth of birds (Heederik et al. 1991, Fiegel et al. 2006). Pathogenic microbes in aerosols can diffuse outside poultry houses through ventilation systems, resulting in their large-scale distribution and causing microbial contamination and long-distance disease transmission. Opportunistic pathogens may not cause problems under normal conditions but can quickly propagate when the livestock and poultry house environment changes and the immunity of the birds decreases (Dutkiewicz et al. 1994, Fiegel et al. 2006). Furthermore, the predominant nonpathogenic microbes can also overload the birds' bodies and decrease their immunity, resulting in increased vulnerability to infectious diseases (Lee et al. 2007, Pavan and Manjunath 2014). Thus the potential harm of microbial aerosols

released from poultry houses cannot be overlooked as an important source of air pollution.

Spring in northern China is characterized by very large temperature differences between day and night. Therefore, most poultry houses are subject to heating insulation by closing windows to provide appropriate warmth for the chickens. Under this condition, the air quality considerably decreases and the microbial aerosol concentration increases in cage systems, which influences the health and meat quality of chickens during normal growth stages. To date, most studies on poultry farming have been focused on the adverse events associated with NH_3 and H_2S , and few studies have been conducted to investigate the potential effects of bacterial communities in $\text{PM}_{2.5}$ and PM_{10} (Choi et al. 2001).

Currently, high-throughput 16S rDNA sequencing is the most commonly used approach for analyzing the structure and diversity of microbial communities. Its strengths include a long read length, high accuracy, and high-throughput capacity (Youssef et al. 2009, Caporaso et al. 2011). In this study, we used the high-throughput 16S rDNA sequencing technique to determine the airborne bacterial community structures in $\text{PM}_{2.5}$ and PM_{10} in broiler houses at different broiler growth stages in the spring. This study aims to illustrate the dynamic pattern of microbial aerosols and potential opportunistic pathogens during the growth cycle of broilers in poultry houses. Our results may provide a basis for developing technologies for air quality control in intensive livestock and poultry farming.

Materials and Methods

Ethics approval

The research protocol was reviewed and approved by the Animal Care and Use Committee (ACUC) of the School of Life Sciences, Ludong University (SKY-ACUC-2017-04).

Sampling sites

Three broiler houses located in the Muping district, Yantai, Shandong Province, China, were selected between March and April 2017, with geographical locations of (37°20'42.19"N, 121°23'01.77"E"), (37°23'91.91"N, 121°24'55.38"E") and (37°22'22.29"N, 121°23'75.97"E"). The broiler houses had windows and a natural light cycle. Chickens in the houses were raised in a closed environment with approximately 20-22 thousand chicks. Before arrival of the broilers, the poultry houses were cleaned and disinfected. All three broiler farms used the closed three-overlap

rearing mode. The buildings were equipped with tunnel ventilation systems, a material pipeline, water curtains, a water pipeline, a fog pipeline, a cement floor, a coal-fired heating furnace and automatic feces transfer. Litter and droppings were regularly cleaned every day. The feedstuff for the three broiler houses was uniformly provided by a breeding/farming enterprise that also provided rearing guidance. Because the broiler houses were set up under the same conditions, the environmental microbe compositions were similar. The three broiler houses were selected randomly, and changes in the microbial structures were analyzed during different broiler growth phases.

PM_{2.5} and PM₁₀ collection

PM_{2.5} and PM₁₀ collection was performed with a ZR-3920 environmental air particulate matter sampler using 9 cm Tissuquartz™ filters (Pall, Port Washington, NY, USA) with typical aerosol retention of 99.9%. Prior to sample collection, the filter was baked and then placed in an environment with a constant temperature and humidity for 48 h. The PM_{2.5} and PM₁₀ concentrations were evaluated using the following formula: $C = (W_1 - W_0) / (t \times F)$, where W_0 and W_1 are the blank film weight and the weight after sample collection, respectively, t is the sampling time, and F is the sampling volume. The sampling device was placed at the center of the poultry houses approximately 1.5 m above the ground. The sampling volume was 100 l/min, and the sampling time was 48 hrs. The sampling was performed in the chickens at the age of 5-7 days (early stage; the PM_{2.5} and PM₁₀ samples were marked as EA.PM2.5 and EA.PM10, respectively), 20-22 days (middle stage; the PM_{2.5} and PM₁₀ samples were marked as MI.PM2.5 and MI.PM10, respectively), and 38-40 days (late stage; the PM2.5 and PM10 samples were marked as LA.PM2.5 and LA.PM10, respectively). The within-house temperature and wind speed were 30-32°C and 0.18-0.20 m/s during the early sampling period, 25-27°C and 0.48-0.51 m/s during the middle stage, and 20-22°C and 1.06-1.10 m/s during the last stage, respectively. Measurements were taken at three time points during different broiler growth stages at the examined chicken houses.

DNA extraction and high-throughput sequencing

Genome DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as conventionally described. PCR amplification was performed using barcode primers specially designed for the 16S V4-V5 region of the 16S ribosome gene (515F: 5'-GTGCCAGCMGCCGCGGTAA-3', 907R: 5'-CCGTCAATTCCTTTGAGTTT-3') (Chen et al.

2016). PCR amplification was carried out using a Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs) under the following conditions: 98°C for 1 min (1 cycle), 98°C for 10 sec/50°C for 30 sec/72°C for 30 sec (30 cycles), and a last step of 72°C for 5 min. PCR products were visualized by 2.0% agarose electrophoresis and purified using a QIAquick Gel Extraction Kit (QIAGEN, Dusseldorf, Germany). The collected samples were categorized into 6 groups based on the sampling period and sites (EA.PM2.5, EA.PM10, MI.PM2.5, MI.PM10, LA.PM2.5 and LA.PM10). A TruSeq® DNA PCR-Free Sample Preparation Kit was used to establish a DNA library according to the manufacturer's instructions. Finally, sequencing was performed on an Illumina HiSeq 2500 platform with a rapid-mode paired-end 250 bp (PE250) protocol (Novogene, Beijing, China).

Data analysis

Paired-end reads were assigned to each specimen according to the barcode sequence before removing their barcode and primer sequences. Afterwards, the trimmed paired-end reads were merged using FLASH software (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>) to obtain splicing sequences, which were termed raw tags. Quality filtering was conducted to eliminate unqualified sequences from the raw reads following the QIIME quality control process (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html). Using this approach, we acquired high-quality clean tags, which were analyzed via the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) by comparing sequences to the Gold database (http://drive5.com/uchime/uchime_download.html) to pick out chimeric sequences and obtain final effective tags. Sequences of these effective tags with an average nucleotide similarity of 97% were clustered into a single operational taxonomic unit (OTU) with the UPARSE software (v7.0.1001, <http://drive5.com/uparse/>). For each OTU, a representative sequence was picked to assign the taxonomic composition with the threshold set as 0.8~1 using the mothur approach and a SILVA (<http://www.arb-silva.de/>) SSU rRNA database. The taxon abundance for each sample was summarized at different taxonomic levels (kingdom, phylum, class, order, family, genus, and species). Alpha and beta diversity indices were analyzed using QIIME 1.7.0. All raw sequences were deposited in GenBank under accession number PRJNA496259.

Statistical analysis

Data was analysed using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA) and SPSS 22.0 (IBM, United

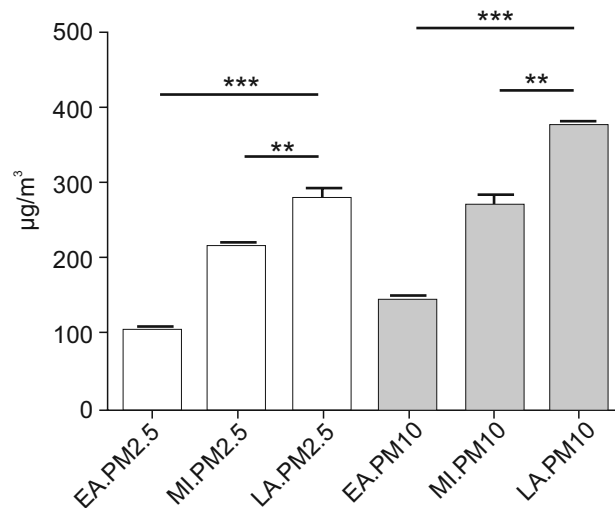


Fig. 1. Alterations in $PM_{2.5}$ and PM_{10} concentrations. Data are expressed as means \pm SDs, *** p <0.001, ** p <0.01 compared between two groups ($n=3$). A difference with p <0.01 or p <0.001 was considered statistically significant. The $PM_{2.5}$ and PM_{10} mean concentrations in the samples collected at the early stage (designed as EA.PM2.5 and EA.PM10) were 104.3, 144.7 $\mu\text{g}/\text{m}^3$, respectively. $PM_{2.5}$ and PM_{10} concentrations in the samples collected at the middle stage (designed as MI.PM2.5 and MI.PM10) were 214.3, 271.0 $\mu\text{g}/\text{m}^3$, respectively. In terms of late stage, the $PM_{2.5}$ and PM_{10} (designed as LA.PM2.5 and LA.PM10) concentrations were 280.7 and 375.7 $\mu\text{g}/\text{m}^3$, respectively.

States). One-way analysis of variance (ANOVA) was employed to analyze the statistical significant differences between sample means. p <0.05 was considered statistically significant, and p <0.01 or p <0.001 was considered extremely statistically significant. All data was analysed in triplicate and expressed as mean \pm standard deviation (SD).

Results

$PM_{2.5}$ and PM_{10} concentrations

The $PM_{2.5}$ and PM_{10} concentrations of the six samples are shown in Fig. 1. Both the $PM_{2.5}$ and PM_{10} sample concentrations within the broiler houses gradually increased with broiler growth. Meanwhile, the $PM_{2.5}$ and PM_{10} concentrations showed significant increases in the late stage compared with those of the middle (p <0.01) and early stages (p <0.001).

Basic statistics of the 16S rDNA gene sequences

A total of 835,082 effective tags were obtained from all of the assayed samples, with approximately 46,393 tags obtained from each sample (range from 31,084 to 61,117). The average length of each effective tag was 373 bp. To investigate the diversity of the samples, effective tags with an identity of 97% were clustered into OTUs. Finally, an average of 527 OTUs was obtained for each sample, and a total of 388 genera was identified. Good's coverage for all samples was >98%, indicating that the majority of microbial phylo-

types were detected. This finding demonstrated that the species richness of the 16S rDNA gene sequence database was high enough to ensure the reliability of further diversity analyses.

Bacterial communities in $PM_{2.5}$

Fig. 2 lists the top 10 bacteria at the phylum and genus levels. At the phylum level, the majority of the bacterial communities in the early growth stage (EA.PM2.5) was *Firmicutes* (91.05%), followed by *Proteobacteria* (2.58%), and *Actinobacteria* (1.56%). For the middle stage (MI.PM2.5), the predominant phyla were *Firmicutes* (97.48%), followed by *Proteobacteria* (0.97%) and *Actinobacteria* (0.56%). The majority of the bacterial communities in the last stage (LA.PM2.5) were composed of *Firmicutes* (46.34%), *Proteobacteria* (34.98%), and *Bacteroidetes* (8.67%). Based on the richness and diversity of the microbial communities, the abundance distribution of the top 35 genera among all samples is displayed at the genus level in the heat map shown in Fig. 3.

At the genus level, the major bacterial genera in the early stage (EA.PM2.5) were *Lachnospirillum* (12.30%), *Lactobacillus* (10.52%), *Faecalibacterium* (8.39%), *Ruminococcaceae* (5.02%), *Subdoligranulum* (4.69%), *Ruminiclostridium* (3.67%), and *Erysipelatoclostridium* (2.42%). The major genera in the middle stage (MI.PM2.5) were *Faecalibacterium* (45.17%), *Lactobacillus* (5.30%), *Subdoligranulum* (4.54%), *Ruminiclostridium* (3.04%), *Lachnospirillum* (2.82%), and *Ruminococcaceae* (2.37%). The major bacterial

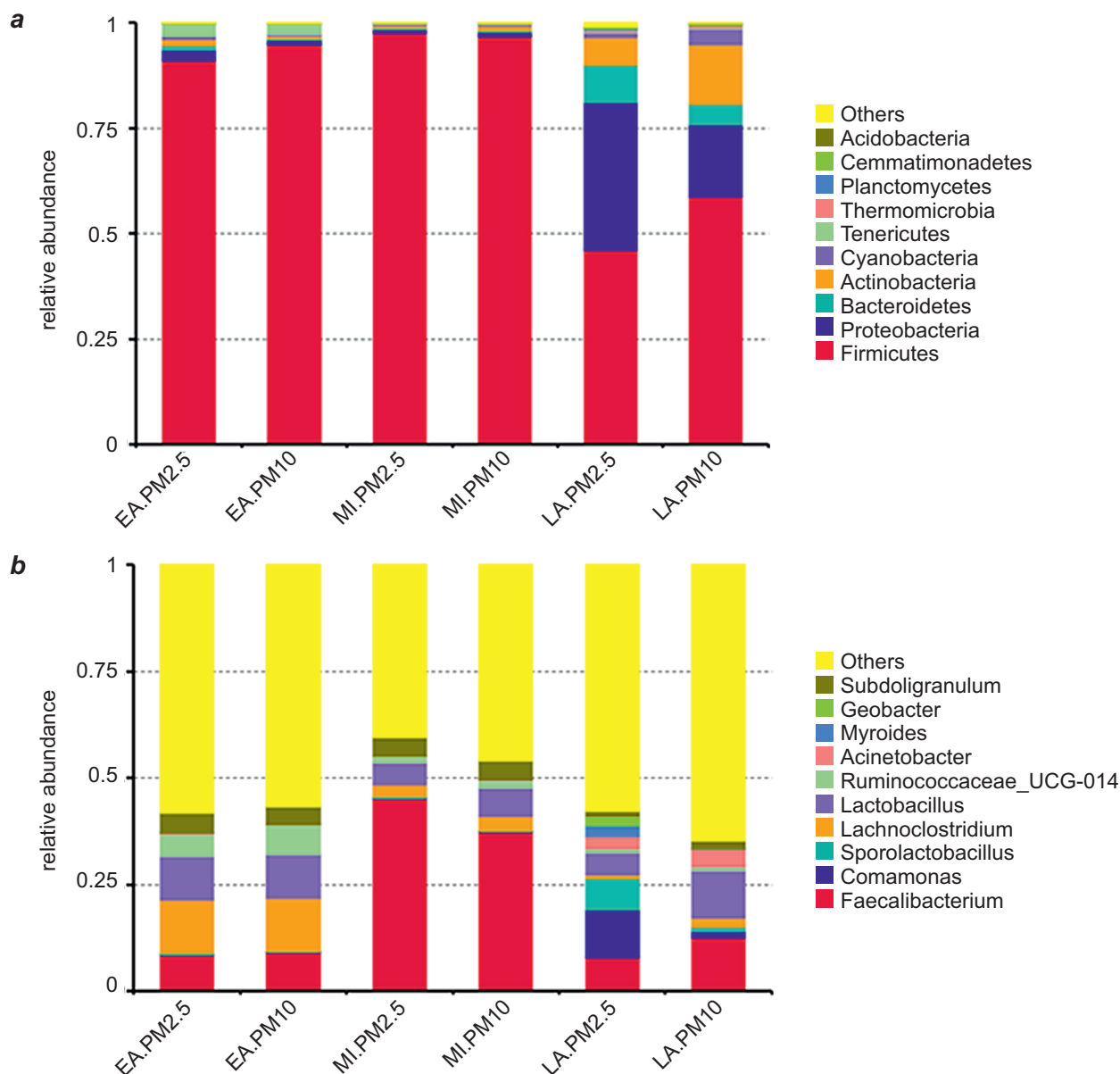


Fig. 2. Relative abundance of the dominant bacteria in different PM samples at phylum (a) and genus level (b). Each colour represents a particular bacterial family. The top 10 abundant taxa are shown. Each bar represents the relative abundance of a group.

genera in the last stage (LA.PM2.5) were *Comamonas* (11.22%), *Faecalibacterium* (7.82%), *Sporolactobacillus* (7.40%), *Lactobacillus* (5.29%), *Acinetobacter* (3.10%), *Myroides* (2.71%), *Bacillus* (2.28%), and *Geobacter* (2.09%).

Bacterial communities in PM₁₀

At the phylum level, the predominant phyla in the early stage (EA.PM10) were *Firmicutes* (94.88%), followed by *Tenericutes* (2.64%) and *Proteobacteria* (1.07%). In the middle stage (MI.PM10), the majority were composed of *Firmicutes* (96.70%), *Proteobacteria* (1.09%) and *Actinobacteria* (1.07%). For the last stage, the predominant phyla in PM₁₀ (LA.PM10) were

Firmicutes (58.96%), followed by *Proteobacteria* (17.05%), *Actinobacteria* (14.31%).

At the genus level, the predominant genera in the early stage (EA.PM10) were *Lachnoclostridium* (12.45%), followed by *Lactobacillus* (10.50%), *Faecalibacterium* (9.15%), *Ruminococcaceae* (6.91%), *Subdoligranulum* (4.08%), *Ruminiclostridium* (3.65%), and *Erysipelatoclostridium* (2.50%). The PM₁₀ collected from the middle stage (MI.PM10) mainly contained *Faecalibacterium* (37.4%), *Lactobacillus* (6.76%), *Subdoligranulum* (4.46%), *Ruminiclostridium* (3.49%), *Lachnoclostridium* (3.25%), *Ruminococcaceae* (2.43%) and *Anaerotruncus* (2.05%). The major bacterial genera in the last stage (LA.PM10) were *Faecalibacterium* (12.41%), *Lactobacillus* (11.13%), *Acinetobacter*

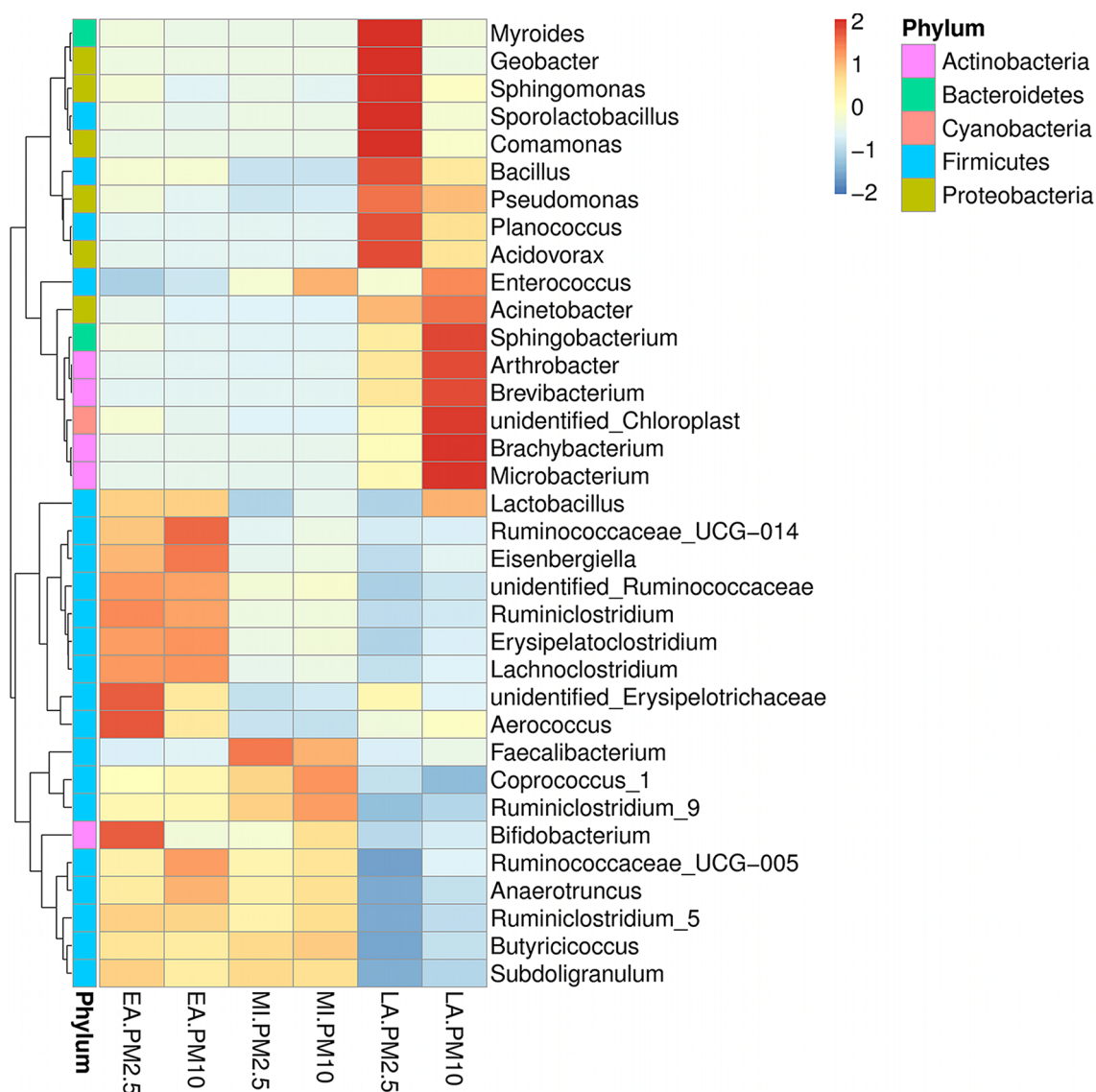


Fig. 3. Heatmap showing changes in microbial communities at the genus level for different development stages of broilers. Sample information is shown crosswise, and species annotation is shown lengthwise. The species cluster tree is shown on the left, and the sample cluster tree is shown at the top.

(4.04%), *Brachybacterium* (3.98%), *Arthrobacter* (2.17%) and *Brevibacterium* (1.98%).

Bacterial community diversity

Alpha diversity was assessed to analyze the complexity of the species diversity in the samples. In the alpha diversity analysis, the rarefaction curves and Simpson index (community diversity) were generated based on 97% similarity. The PM_{2.5} and PM₁₀ rarefaction curve analysis (Fig. 4) of the observed numbers of species indicated that the last stage (LA.PM2.5 and LA.PM10) had the highest level of bacterial diversity among the samples assayed. However, we found no significant differences in Simpson diversity among the PM samples in the broiler houses at the different broiler growth stages ($p > 0.05$, Fig. 5).

In the beta diversity analysis, principal coordinates analysis (PCoA) was used to extract the most important elements and structures from the multidimensional data through calculation of a series of eigenvalues and eigenvectors. The combined PCoA (Fig. 6) and multiresponse permutation procedure (MRPP) analysis results (which were used to determine whether a significant difference existed in the microbial community structure between groups) demonstrated that the bacterial community structure of each group had adequate differences and that the differences between groups were greater than the differences within groups, although the differences were not significant ($p > 0.05$).

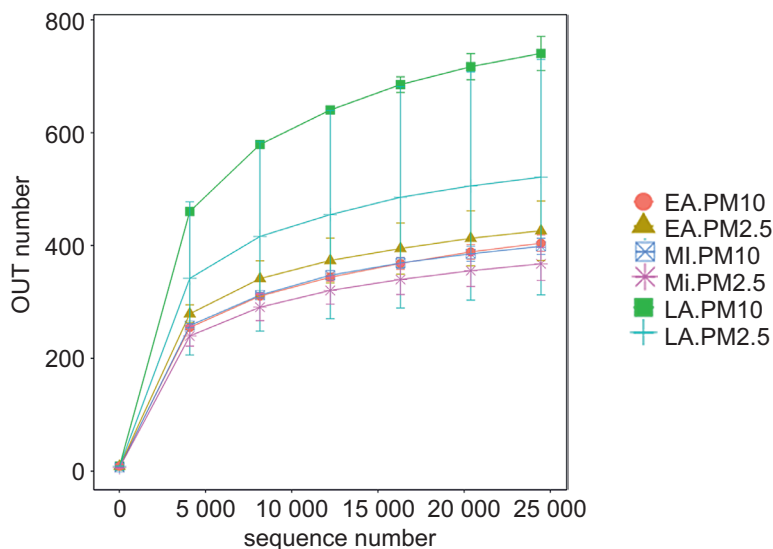


Fig. 4. Rarefaction curves of 16S rDNA sequences for bacterial diversity. OTU: operational taxonomic units.

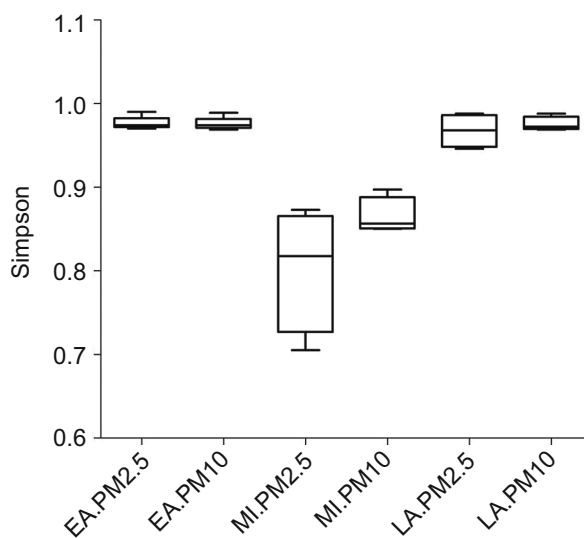


Fig. 5. Boxplot analysis of the Simpson index. Each box plot represents the indicated richness estimator.

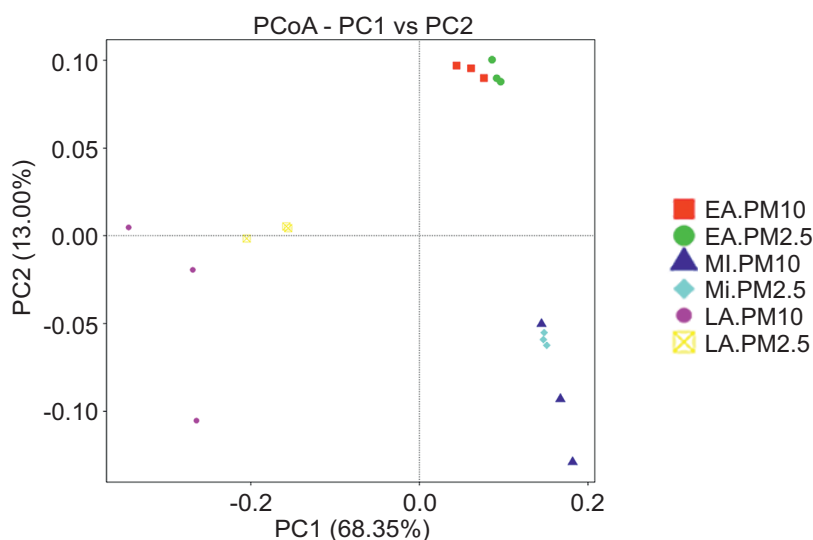


Fig. 6. PCoA analysis of bacterial communities in the sample groups. The abscissa represents one principal component, and the ordinate represents another principal component. The percent values indicate the contributions of the principal components to sample differences. Each dot in the plot represents one sample, and all samples in the same group are represented by the same colour. PC1 represents one principal component, PC2 represents another principal component, and the percentages represent the contribution of the principal components to the sample differences.

Discussion

In this study, microorganisms carried by airborne PM within broiler houses in spring under intensive feeding conditions at different broiler growth stages were examined, and the diversity of the bacterial communities in PM_{2.5} and PM₁₀ was analyzed. Our research showed that the PM_{2.5} and PM₁₀ concentrations increased with the age of the chickens in the houses. We reasoned that because the poultry houses needed to be thoroughly vacated, cleaned and sterilized before arrival of the broilers, the initial PM_{2.5} and PM₁₀ concentrations were at the lowest levels or even lower than those of the surrounding environment. As the broilers grew, their daily activities, excrement, and density increased, all of which might have elevated the PM_{2.5} and PM₁₀ concentrations in the poultry houses.

The high-throughput 16S rDNA sequencing analysis detected 27 phyla, 55 classes, 106 orders, 196 families, and 388 genera. This finding illustrated remarkable airborne microbial diversity in PM_{2.5} and PM₁₀ inside the broiler houses. The predominant phyla were *Firmicutes* and *Proteobacteria*, which was in line with a previous study (Hong et al. 2012). A total of 367 genera in PM_{2.5}, including 42 potential or opportunistic pathogens, and 371 genera in PM₁₀, including 42 potential or opportunistic pathogens, were detected in the poultry houses. The bacterial diversity analysis showed negligible significant differences between PM_{2.5} and PM₁₀ during the same period. As the chickens grew, the number of observed species of the atmospheric microbiota increased and reached the highest level during the late growth stage of the broilers. This result indicated that the airborne microorganisms increased as the broilers grew, which was in agreement with other reports (Vučemilo et al. 2007, Oppliger et al. 2008). This change may be due to the growth and metabolism of the broilers. Recent studies have revealed that many microbes are involved in the biological processes of digestion, absorption, and metabolism in broilers (Berry and Reinisch 2013, Li et al. 2014). Moreover, the lack of significant differences in the Simpson diversity and the PCoA analysis of the bacterial communities in the PM samples showed that, although the airborne microbial community structure in the broiler house did exhibit some changes during the broiler growth cycle, the differences were not significant. Although some differences in abundance were observed for the same bacterial genera, the majority of the bacterial communities carried by the PM were the same across the different growth stages.

The genera in the broiler houses at different growth stages in spring included *Acinetobacter*, *Pseudomonas*, *Enterococcus*, *Microbacterium*, etc., all of which con-

tributed some pathogens or opportunistic pathogens. For example, certain species in *Acinetobacter* are important pathogens, such as *A. baumannii*, which can infect people and poultry and is often isolated from chicken houses (Hinton et al. 2004). *Pseudomonas aeruginosa* can cause infections in chickens that lead to septicemia and death (Cachia and Hodges 2003). Strains of *Enterococcus* can cause many diseases in humans and birds, such as *E. faecalis*, which can increase the incidence of diseases in broilers at various ages (Hammerum et al. 2000). *Microbacterium* is often detected in poultry, eggs and meat; a few strains of this genus can affect human and bird health (Kraft et al. 1966). These pathogens and opportunistic pathogens can contaminate the farming environment when air circulation is inadequate, resulting in an increased occurrence of diseases. This finding implies that, for poultry and livestock farming in spring, keeping the environment warm and enforcing ventilation and disinfection are important for the prevention of airborne diseases in poultry houses.

Previously, we examined the aerosol concentrations and bacterial community structures of closed cage broiler houses in winter (Jiang et al. 2018). A total of 35 phyla and 537 genera were detected during the entire broiler rearing process in winter, which was higher than the results from this study (27 phyla and 388 genera); additionally, the types of opportunistic pathogens in the aerosols in the winter were greater than those in the spring. The relative abundances of many pathogenic bacteria in winter were larger than those in spring at the same broiler growth stage, such as *Escherichia* and *Pseudomonas*, which are important pathogens in the poultry industry. Some studies have indicated that PM and atmospheric microbes in poultry houses can be affected by many factors, including seasonal variation, bird activity, air temperature, relative humidity, and ventilation rates (Cambrá-Lopez et al. 2011). Farmers tend to pay more attention to keeping broilers warm and neglect ventilation of poultry houses due to the cold climate in winter. This situation will result in air quality deterioration in poultry houses and provide conditions for many bacteria to grow. This study should provide a reminder that ventilation is an important factor for improving the indoor environment of broiler farming.

The health of poultry under intensive farming is closely associated with the living environment. In a closed environment, air quality in houses gradually decreases as chicks grow. In this study, we observed that as the broilers grew, the PM_{2.5}, PM₁₀ and airborne microorganisms gradually increased in the houses. The elevated levels of airborne PM and microbes can potentially cause respiratory or intestinal diseases

in poultry. Thus, as the broilers grow, appropriate measures, including timely removal of filth, maintenance of suitable humidity and ventilation, and disinfection of broilers (i.e., disinfecting the broiler houses while the chickens are present), are required to control the airborne PM and microbe concentrations in the broiler houses and to decrease the occurrence of opportunistic diseases (i.e., diseases caused by opportunistic pathogens), which will provide a better farming environment for poultry. In addition, the 16S rDNA sequencing technique can accurately classify microbes to the genus level but not to the species level (Janda et al. 2007). Therefore, to confirm these results, bacteria must be isolated and identified using classical microbiological methods (e.g., biochemical tests). We are planning to continue relevant studies in the future.

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