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Composition and distribution analysis of bioaerosols under different environmental conditions

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1 **TITLE:**

2 Composition and Distribution Analysis of Bioaerosols Under Different Environmental Conditions

3

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29 **SUMMARY:**

30 Understanding the biological composition of environmental particulate matter is important for
31 the study of its significant impacts on human health and disease spread. Here, we used three
32 types of bioaerosol sampling methods and a biological analysis of airborne microbes to better
33 explore airborne microbial communities under different environmental conditions.

34

35 **ABSTRACT:**

36 Variable microorganisms in particulate matter (PM) under different environmental conditions
37 may have significant impacts on human health. In this study, we described a protocol for
38 multiple analyses of the biological compositions in environmental PM. Five experiments are
39 presented: (1) PM number monitoring by using a laser particle counter; (2) PM collection by
40 using a cyclonic aerosol sampler; (3) PM collection by using a high-volume air sampler with
41 filters; (4) culturable microbes collection by the Andersen six-stage sampler; and (5) detection of

42   biological composition of environmental PM by bacterial *16S rDNA* and fungal *ITS* region
43   sequencing. We selected hazy days and a livestock farm as two typical examples of the
44   application in this protocol. In this study, these two sampling methods, cyclonic aerosol sampler
45   and filter sampler, showed different sampling efficiency. The cyclonic aerosol sampler
46   performed much better in terms of collecting bacteria, while these two methods showed the
47   same efficiency in collecting fungi. Filter samplers can work under low temperature conditions
48   while cyclonic aerosol samplers have a sampling limitation for temperature. A solid impacting
49   sampler, such as an Andersen six-stage sampler, can be used to sample bioaerosols directly into
50   the culture medium, which increases the survival rate of culturable microorganisms. However,
51   this method mainly relies on culture while more than 99% of microbes cannot be cultured. DNA
52   extracted from the culturable bacteria collected by the Andersen six-stage sampler and samples
53   collected by cyclonic aerosol sampler and filter sampler were detected by bacterial *16S rDNA*
54   and fungal *ITS* region sequencing. All the methods above may have wide application in many
55   fields of study, such as environmental monitoring and airborne pathogen detection. From these
56   results, we can conclude that these methods can be used under different conditions and may
57   help other researchers further explore the health impacts of environmental bioaerosols.
58
59   **INTRODUCTION:**
60   In natural environments, various kinds of microorganisms exist in bioaerosols, including fungi,
61   bacteria, viruses and other microorganisms[1]. Airborne microorganisms, which can be emitted
62   from some human activities such as animal feeding operations in livestock farms, are the
63   important contents of the atmospheric environment[2]. These microorganisms may not only play
64   important roles in the atmospheric environment but also have significant impacts on human
65   health and diseases spread.
66
67   As an important way of spreading diseases, microbial aerosols have attracted wide attention
68   throughout the world. In recent studies, many human diseases were found to be associated
69   with the complex composition of environmental particulate matter (PM) in different locations,
70   such as chemical factories, livestock farms and smoggy cities[3,4]. The biological composition of
71   PM may contribute to some respiratory and cardiovascular diseases in PM-exposed humans[5].
72   Different body regions, such as the mucosa, skin, digestive tract and respiratory tract, can be
73   potential targets of microbes attached to PM[6,7]. An increased risk of lung cancer might be
74   caused by prolonged exposure to $PM_{2.5}$ [8].
75
76   Airborne bacteria have been surveyed at various locations in several countries around the
77   world, including in subway stations, veterinary hospitals, slaughterhouses, composting facilities,
78   tanneries, milk-processing facilities, coal mines, dental clinics and indoor environments[9-17],
79   generating a large number of reports about biological aerosols. Crowded places associated with
80   campuses, livestock farms and large cities during hazy days are three particularly important
81   public conditions for which we need to explore the connections between human health and the
82   potential effects of PM exposure. Furthermore, during winter days in the northern cities of

83 China, high PM$_{2.5}$ values may affect human health. Although PM$_{2.5}$ can generate toxic effects by
84 targeting respiratory surfaces and dissolving into blood[18], it is still not clear whether and how
85 the microbes attached to PM$_{2.5}$ could potentially affect human health[19,20]. Livestock farms are
86 one of the main sources of PM and microbial aerosols in the air. The large numbers of
87 pathogens, such as influenza virus and *Brucella melitensis*, that are carried by aerosols in the
88 fields around livestock farms are important factors causing respiratory diseases in both
89 livestock and poultry workers.
90
91 In this study, we explored multiple types of analyses of bioaerosols, including PM number
92 monitoring, bioaerosol collection and biological composition analysis. Air samples were
93 collected by a cyclonic aerosol sampler, a high-volume air sampler with filters and an Andersen
94 six-stage sampler. Then, the samples collected by these three samplers were analyzed by
95 biological analysis including bacterial *16S rDNA* and fungal *ITS* sequencing to determine their
96 biological compositions. Herein, we show representative results from the bioaerosol samples
97 collected during Beijing hazy days and from livestock farms indicating that bioaerosols might
98 have great impacts on human and animal health. The comparison between liquid and filter
99 sampling methods were also explored in this study mainly based on the data from 16S DNA and
100 fungal ITS sequencing.
101
102 **PROTOCOL:**
103
104 **1.   PM Number Monitoring**
105
106 1.1. Use an airborne laser particle counter (see **Table of Materials**) to determine the total PM
107 number. Collect the PM by the air sampling port on the top of the airborne laser particle
108 counter.
109
110 NOTE: This particle counter is an automation equipment and it can work independently when
111 the program including sampling time, interval, collection times, *etc.* has been set on its touch
112 screen.
113
114 1.1.1.   Equip the instrument with a sensor to monitor temperature and relative humidity.
115 Measure and record temperature and relative humidity data simultaneously every 5 min.
116
117 1.1.2.   In total, measure and record 6 different particle size classes (0.3-0.5 μm, 0.5-0.7 μm,
118 0.7-2.5 μm, 2.5-5 μm, 5-10 μm and > 10 μm) simultaneously every 5 min. Measure 4 other
119 particle size classes (0.3-0.5 μm, 0.5-1 μm, 1-3 μm and ≥ 3 μm).
120
121 1.1.3.   Collect the air samples though the sampling hole on the top of the sampler and use the
122 test modules inside the sampler to measure the particle size of each PM. Then the data is
123 stored automatically. All the processes above can be performed automatically after the relative

124 parameters, including sampling time and counting range, are set though the mini touch
125 displayer of the airborne laser particle counter.

126

127 NOTE: This particle counter is an automation equipment and it has test modules that count the
128 number of PM of different particle size classes.

129

130 1.2. To assess experimental standard error, ensure that different particle size classes are
131 counted with at least 15 replications. Ensure that readings are stored in the internal memory of
132 the instrument and subsequently analyzed.

133

134 1.2.1.   Ensure that analysis of the primary data includes PM number concentration, the
135 ANOVA test and the percentage of PM in each size class. For example, as shown in **Figure 1A**,
136 the PM number concentrations in December (237.6 particles/cm^3) were significantly higher
137 than those in October (average: 110.2 particles/cm^3) (ANOVA; P-value < 0.05). **Figure 1B** shows
138 that the number of particles smaller than 3 µm accounted for more than 99% of the total
139 number.

140

141 1.2.2.   Use the laser particle counter to monitor the PM number concentrations and store the
142 data automatically. Use a USB flash disk to export data in the computer and perform the
143 ANOVA test.

144

145 **2.   PM Collection by Cyclonic Aerosol Samplers**

146

147 2.1. Perform the PM sampling in an open area without any nearby major pollution sources at ~2
148 m above the ground.

149

150 2.1.1.   Record the position of the sampling site. For example, the campus of Beijing Institute
151 of Technology is the following: 39°57'51.0'' N; 116°19'38.5'' E.

152

153 2.2. Use a cyclonic aerosol sampler to collect air samples. Use a sampler flow of 323 L/min, and
154 a collection time of 6 h.

155

156 NOTE: The sampler flow rate is fixed and cannot be changed. The collection time is controlled
157 by manual time-keeping as there is only a **Start** and **Stop** button on this sampler.

158

159 2.2.1.   Use sterile water to wash the inside of the cyclonic aerosol sampler 3 times before
160 collection, and use the automatic cleaning function 3 times by continuously pressing the collect
161 button 3 times.

162

163 2.2.2.   Press the collect button to start sampling and press the pump button to stop sampling.
164 Put the sampler on a shelf or floor with no one holding it in place.

165
166 2.2.3.   Preserve all samples in the dark at -20 °C until the subsequent analyses.
167
168 **3.   PM Collection by Filters**
169
170 3.1. Perform the PM sampling in an open area without any nearby major pollution sources at ~2
171 m above the ground.
172
173 3.1.1.   Record the position of the sampling site. For example, the campus of Beijing Institute
174 of Technology is as follows: 39°57'51.0'' N; 116°19'38.5'' E.
175
176 3.2. Collect the PM samples on 20.32 × 25.4 cm^2 filters (see **Table of Materials**) by using a high-
177 volume air sampler (see **Table of Materials**) at a flow rate of 1000 L/min. Set the flow rate and
178 collection time by relative auto-programming.
179
180 3.2.1.   Preserve all sampled filters in the dark at -20 °C until the subsequent analyses.
181
182 **4.   Biological Composition Analysis**
183
184 4.1. For biological composition analysis, use each liquid sample from the cyclonic aerosol
185 sampler or filter sample for DNA extraction by a multisource DNA extraction Kit (see **Table of**
186 **Materials**) according to the manufacturer's protocols.
187
188 4.1.1.   For samples from the high-volume air sampler with filters, use 1/8 of each filter sample
189 for DNA extraction. Put the filter into a 50 mL tube with the sample facing inward and the back
190 facing towards the tube wall. Add 10 beads in the central sites towards the side of the filter
191 containing the sample.
192
193 4.1.2.   Vortex the tube for 15 min at room temperature. Pipette the liquid in the tube into a
194 clean 50 mL centrifuge tube to continue the DNA extraction process.
195
196 4.2. Extract the DNA from the sample by the processes as follows.
197
198 4.2.1.   Centrifuge the bacteria sample at 2000 x g for 5 min, remove the supernatant, and
199 suspend bacteria in 2 mL of sterile PBS buffer.
200
201 4.2.2.   Collect the suspension of bacteria in a 2 mL centrifuge tube and then centrifuge at
202 2000 x g for 5 min to discard the supernatant solution.
203
204 4.2.3.   Add 350 µL of sterile PBS buffer containing the suspended bacteria to the solution.
205

206   4.2.4.   Add 0.8 mL of RNase A.

207

208   4.2.5.   Add 150 µL of Buffer CL and 8 µL of proteinase K, and mix immediately by vortex
209   shaking for 1 min. After brief centrifugation at 1000 x g for 30 s (no residues on the wall), put
210   the centrifugal tube in 56 °C water for 10 min.

211

212   4.2.6.   Add 350 µL of Buffer PD, and mix for 30 s and then centrifuge for 10 min at 12000 x g.

213

214   4.2.7.   Place the DNA preparation tube in a 2 mL centrifuge tube, and transfer the mixture
215   made in step 4.2.6 to the preparation tube. Then centrifuge for 1 min at 12000 x g.

216

217   4.2.8.   Discard the filtrate, put back the contents of the preparation tube into the original 2 mL
218   centrifuge tube, and add 50 µL of Buffer W1. Centrifuge the mixture for 1 min at 12000 x g.

219

220   4.2.9.   Discard the filtrate, put back the contents of the preparation tube into the original 2 mL
221   centrifuge tube, and add 700 µL of Buffer W2. Centrifuge the mixture for 1 min at 12000 x g.
222   Repeat this step to wash again with 700 µL of Buffer W2.

223

224   4.2.10.   Discard the waste liquid, and put back the contents of the preparation tube into the
225   original 2 mL centrifuge tube. Centrifuge the mixture for 1 min at 12000 x g.

226

227   4.2.11.   Put the contents of the DNA preparation tube into another clean 1.5 mL centrifuge
228   tube, and add 100 µL of eluent or deionized water to the middle of the membrane in the
229   preparation tube (deionized water or eluent was heated to 65 °C). Allow the mixture to stand at
230   room temperature for 1 min, and Centrifuge the mixture for 1 min at 12000 x g.

231

232   4.3. Perform quantitative real-time polymerase chain reaction (Q-RT-PCR) to quantify the
233   relative abundances of bacteria and fungi on the sampling filters.

234

235   4.3.1.   Use primers as follows: for bacterial *16S rDNA*, 515F (5'- GTG CCA GCM GCC GCG GTA
236   A-3') and 806R (5'- GGA CTA CHV GGG TWT CTA AT-3'); and for the fungal *ITS*, ITS1 (5'-TCC GTA
237   GGT GAA CCT GCG G-3') and ITS1 (5'-GCT GCG TTC TTC ATC GAT GC-3').

238

239   4.3.2.   Run the Q-RT-PCR assays on a Real-Time PCR System (see **Table of Materials**). RT-PCR
240   condition: predegeneration, 95 °C, 10 min; degeneration, 95 °C, 15 s; anneal and extension,
241   60 °C, 1 min; 40 cycles from degeneration to anneal and extension.

242

243   4.4. For bacterial and fungal community structure analysis, amplify the V1–V3 region of the
244   bacterial *16S rDNA* and the *ITS* region of the fungal rRNA operon by PCR.

245

246   4.4.1.   Use the primers as follows: for bacteria, V1-9F (5'-CCT ATC CCC TGT GTG CCT TGG CAG
247   TCT CAG ACG AGT TTG ATC MTG GCT CAG-3') and V3-541R (5'-CCA TCT CAT CCC TGC GTG TCT
248   CCG ACT CAG-barcode-ACW TTA CCG CGG CTG CTG G-3'); and for fungi, ITS-3F (5'-CCT ATC CCC
249   TGT GTG CCT TGG CAG TCT CAG CAC ATC GAT GAA GAA CGC AGC-3') and ITS-4R (5'-CCA TCT CAT
250   CCC TGC GTG TCT CCG ACT CAG-barcode-GCT CCT CCG CTT ATT GAT ATG C-3').

251

252   4.4.2.   Ensure that the 20 µL mixture of PCRs included 2 µL of 2.5 mM dNTPs, 4 µL of 5×
253   FastPfu Buffer, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of
254   template DNA (see **Table of Materials**).

255

256   4.4.3.   Use the PCR program as follows: 94 °C for 5 min; followed by 10 cycles of 94 °C for 30 s,
257   55-60 °C for 45 s, and 72 °C for 90 s; 20 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s;
258   and a final extension at 72 °C for 5 min.

259

260   4.5. Perform DNA purification, DNA quantification and pyrosequencing as described in a
261   previous study [21].

262

263   **5.   Bioaerosol Sampling and Cultivation**

264

265   5.1. Use an international standard Andersen six-stage sampler (see **Table of Materials**) to
266   sample culturable airborne bacteria and fungi at a flow rate of 28.3 L/min. Use a sampling time
267   of 35 min. Put the culture plate in each stage of the sampler. Sufficiently disinfect the sampler
268   with 75% ethyl alcohol after each sampling.

269

270   NOTE: The sampler flow rate is fixed and the collection time is controlled by manual time-
271   keeping.

272

273   5.1.1.   Sample the six stages with the Andersen six-stage sampler, which is defined by the
274   aerodynamic diameters of the airborne particles, including stage VI (0.65–1.1 µm), stage V (1.1–
275   2.1 µm), stage IV (2.1–3.3 µm), stage III (3.3–4.7 µm), stage II (4.7–7.0 µm) and stage I (≥7.0
276   µm).

277

278   5.1.2.   Collect the bacterial particles and deposit onto the culture plate in each stage
279   containing Soybean-Casein Digest Agar (see **Table of Materials**). There is a container with many
280   air intakes on the top in each stage of the sampler.

281

282   5.1.3.   Culture the airborne bacteria collection plates at 37 °C for 24-48 h; then, count the
283   colony numbers of bacteria on the sample dish for each stage.

284

285   5.2. To prevent the particles collected by the Andersen six-stage sampler from overlapping,
286   correct the number of colonies at each sampler level by the following formula:

287 $$Pr = N\left(\frac{1}{N} + \frac{1}{N-1} + \frac{1}{N-2} + \dots + \frac{1}{N-r+1}\right)$$

288 where Pr: corrected number of colonies at each level,
289 N: number of sampling holes of the sampler at all levels (400), and
290 r: the actual count of colonies.

291

292 5.3. Calculate the unit of colonies per m^3 of air as follows:

293 $$C = \frac{N_1 + N_2 + N_3 + N_4 + N_5 + N_6}{t \times F} \times 1000$$

294 where C: concentration (CFU/m^3),
295 N_1-N_6: the corrected number of colonies at each level,
296 T: sampling time (min), and
297 F: sampling flow rate (28.3 L/min).

298

299 5.4. Use methods from Steps 5.1 - 5.3 to study the bioaerosol in livestock farm, including four
300 types of piggeries.

301

302 NOTE: The livestock farm is located in Changchun, China. The central position 2 m above the
303 ground in each piggery was selected as sampling location.) After culture of bacteria, treat all
304 colonies in the plates as described in Steps 6.1 - 6.2.

305

306 **6.   Identification of Culturable Bacteria**

307

308 6.1. After 48 h or 72 h of culturing, place the bacteria into a 2 mL centrifuge tube. Extract the
309 DNA of these bacteria or fungi by using a multisource DNA extraction Kit (see **Table of**
310 **Materials**). The DNA extraction process is described in section 4.2.

311

312 6.2. Use 200 µL DNA extraction sample for *16S rDNA* sequencing. Use the same processes as
313 described in Steps 4.2, 4.3 and 4.4 on biological composition analysis.

314

315 **REPRESENTATIVE RESULTS:**
316 In this study, we performed an assessment of the overall PM distribution and a comprehensive
317 analysis of the bioaerosols in a dairy farm from September to December. Many environmental
318 factors contribute to the distribution of aerosol particles. We studied the concentration and size
319 distributions of PM in a cow house by using a TSI laser particle counter. As shown in **Figure 1A**,
320 the concentration of aerosol particles was highest in December and lowest in October, which
321 might be caused by changes in temperature and humidity (**Table 1**). The concentration of
322 inhalable aerosol particles (0.3-3.0 µm) accounted for more than 99% of the total particle
323 concentration (**Figure 1B**), and the particles in this range could reach the deep respiratory tract,
324 causing serious hazards for humans and animals.

325

326   Biological composition analysis of samples can be performed by DNA extraction, bacterial *16S*
327   *rDNA* and fungal *ITS* region sequencing instead of microorganism culture. From the biological
328   analysis of bioaerosol samples collected by using a cyclonic aerosol sampler or a high-volume
329   air sampler with filters, we can preliminarily compare the efficiencies of these two methods in
330   collecting bacteria and fungi. **Figure 2** showed the analysis results of the bioaerosol samples
331   collected during Beijing hazy days in the campus of Beijing Institute of Technology in December
332   20, 2016. For bacteria collection, the results indicated that the cyclonic aerosol sampler
333   collected many more genera than the high-volume air sampler with filters (**Figure 2A**). For fungi
334   collection, these samplers showed equal collection efficiencies and almost the same genus
335   abundances (**Figure 2B**). From the results presented in **Figure 2**, we were able to measure the
336   different collection efficiencies of these two methods for bacteria and fungi. For bacteria
337   collection, the cyclonic aerosol sampler performed much better than the high-volume air
338   sampler with filters because the samples from the former showed a higher genus abundance
339   (**Figure 2A**). However, the fungal sequencing analysis of the two samples from different
340   sampling methods showed nearly identical community structures (**Figure 2B**).

341

342   We studied airborne culturable bacteria by using an Andersen six-stage sampler. As shown in
343   **Figure 3**, the colony numbers of culturable bacteria for stage I-VI particles was reduced. Stage I
344   particles (particle size > 8.2 μm) had the highest numbers of culturable bacteria colonies. The
345   percentage of stage I colonies in four different types of piggeries including farrowing house,
346   pregnant sow house, fattening house and weaning house was 33%, 30%, 26% and 34%,
347   respectively. The percentage of stage II colonies in four different types of piggeries was 20%,
348   22%, 19% and 20% respectively. The percentage of stage III colonies in four different types of
349   piggeries was 18%, 18%, 18% and 19% respectively. The percentage of stage IV colonies in four
350   different types of piggeries was 17%, 16%, 16% and 16% respectively. The percentage of stage V
351   colonies in four different types of piggeries was 10%, 10%, 14% and 6% respectively. Stage VI
352   particles (particle size < 1.0 μm) had the lowest numbers of culturable bacteria colonies. The
353   percentage of stage VI colonies in the four different types of piggeries was 3%, 5%, 6% and 5%,
354   respectively.

355

356   The air samples were collected in four different types of piggeries by using an Andersen six-
357   stage sampler and then cultured under suitable conditions. The whole-genome DNA of the
358   culturable bacteria collected from each particle stage was extracted and detected by bacterial
359   *16S rDNA* and fungal *ITS* region sequencing. A total of 91 genera and 158 species of bacteria
360   were identified in the culturable bacteria in piggeries. The culturable bacteria community
361   structures in four different types of piggeries, including farrowing house, pregnant sow house,
362   fattening house and weaning house are shown in **Figure 4** with data from stage I to stage VI. The
363   content of different predominant bacterial genera is not the same among different piggeries.

364

365   **FIGURE LEGENDS:**

Figure 1. The concentration and size distribution of PM in four different months. (A) Boxplot of PM number during the study period. Each boxplot includes the Maximum, minimum, median, two quartiles and abnormal value of the database. (B) Average size distribution maps of PM. There were eighty cows in the house from September to December. The percentage of PM (≥ 3 μm) in four months (September, October, November and December) was 0.005, 0.005, 0.002 and 0.002 respectively.

Figure 2. Biological analysis of bioaerosol samples collected by two samplers. (A) and (B) show the abundances of bacterial or fungal genera in bioaerosol samples obtained with different collection methods. Wetted-Wall Air Sampler represents the cyclonic aerosol sampler. Quartz Filters represents the high-volume air sampler with filters.

Figure 3. Average hierarchical distribution maps of culturable bacteria in four types of piggeries. The four types of piggeries are farrowing house, pregnant sow house, fattening house and weaning house. S1 to S6 represent the six particle stages (I to VI) collected by the Andersen six-stage sampler. The stages were defined by the aerodynamic diameters of the airborne particles, including stage VI (0.65–1.1 μm), stage V (1.1–2.1 μm), stage IV (2.1–3.3 μm), stage III (3.3–4.7 μm), stage II (4.7–7.0 μm) and stage I (7.0 μm).

Figure 4. Bacterial community structures with different abundances in the air samples. The air samples were collected in four different types of piggeries by using an Andersen six-stage sampler and then cultured under suitable conditions. The whole-genome DNA of the culturable bacteria in each particle stage collected by the Andersen six-stage sampler was extracted and detected by bacterial *16S rDNA* sequencing. The numbers 1 to 6 in each type of piggery represent particle stages I to VI measured by the Andersen six-stage sampler. The text on the right side includes the genus name for each bacterium.

DISCUSSION:
In this study, we provided some representative results that were obtained during hazy days and in livestock farms. The results from bioaerosol samples taken during Beijing hazy days facilitated a better understanding of the biological compositions of the biological composition of PM without PM present during Beijing hazy days. The results from samples taken from livestock farms will also provide basic data for environmental air quality control in piggeries and a theoretical foundation and technical support for healthy breeding and safe production in livestock farms. Many environmental factors, such as temperature, humidity and wind speed, might contribute to the distribution of aerosol particles in the cow house (**Figure 1**). Previous studies have revealed that the PM in livestock farms mainly comes from the fodder, feces, fur and feather, which may be related to the animal activities. The environmental factors can affect not only animal activities, but also the aggregation and diffusion of PM in a relatively closed cow house. Therefore, we detect different concentrations and size distributions of PM in four different months. Besides, the sanitary condition, feeding method and animal activity were

407   different between the four types of piggeries, which might also affect their community structure
408   of culturable bacteria in the air (**Figure 4**).
409
410   However, in this study, we mainly focused on the available methods that we could use to study
411   the composition and distribution of bioaerosols under different environmental conditions.
412   Compared with other microbial samples, those of airborne microorganisms have very low
413   concentrations and are mixed with a large number of impurities, such as inorganic dust
414   particles, which introduce certain difficulties during the collection and detection of such
415   microorganisms[21]. Therefore, appropriate methods of collection and detection should be
416   selected for microbial aerosols. The collection of microbial aerosol samples is generally
417   performed by using the precipitation method or specialized equipment to collect the
418   microorganisms in liquid, semi-solid or solid sampling medium[22-24]. Then, some corresponding
419   technical treatment and specific testing and analysis are subsequently carried out[25]. The
420   sampling medium should keep microorganisms intact to reduce the error associated with
421   detection and analysis[26]. However, different aerosol microorganism samplers have different
422   effects on the integrity of samples due to their different sampling principles and media. People
423   have designed many kinds of bioaerosol samplers that use different sampling principles, such as
424   inertial impaction, filtration resistance, and electrostatic precipitation[27].
425
426   Impacting samplers can push airborne PM into the sampling medium at high speed by using
427   extraction equipment. There are two types of impacting samplers: solid and liquid. Solid
428   impacting samplers can be used to sample bioaerosols at a low concentration and can be almost
429   impervious to air flow[28]. Aerosol particles of different sizes can be preliminarily screened, and
430   microbes can be sampled directly into the culture medium, which increases the survival rate of
431   culturable microorganisms[10]. Due to inertial impact, microbial aerosol particles easily collide at
432   the same site, and colonies may overlap easily after culture. At present, the most common solid
433   impacting sampler is the Andersen-6 microbial aerosol sampler. In this study, we used an
434   Andersen six-stage sampler to study the culturable bacteria distributed in airborne PM of
435   different sizes.
436
437   Cyclonic aerosol samplers use cyclones to spiral air at high speed into a cylinder or cone.
438   Bioaerosol particles can be separated from the airflow by centrifugal force, which means that
439   microbes can bump into the inner wall of the sampler and then be collected by the sampling
440   buffer. This method is convenient and can be used for long sampling times in large flow and
441   sampling operations. However, this method cannot be implemented at low temperatures
442   because the operation is dependent on liquid. The cyclonic aerosol sampler used in this study is
443   a cyclonic wetted-wall aerosol sampler that can extract and transfer airborne pathogens and
444   particles from sampled air into a small volume of water for analysis[29].
445
446   Filter samplers can work under low temperature conditions and can sample particles above a
447   certain size. However, they have a great impact on microbial activity and are prone to damage,

448 which will greatly influence subsequent studies on sampling activity. In this study, bioaerosol
449 samples from Beijing hazy days were collected by using both a high-volume air sampler with
450 filters and a cyclonic aerosol sampler. The aim of this experiment was to analyses the biological
451 composition of PM without the separation and culturing of airborne microbes. Therefore, these
452 two sampling methods were suitable for this study. For the cyclonic aerosol sampler method,
453 airborne microbes at a low concentration can be easily extracted into the running buffer and
454 then can be analyzed conveniently without the additional treatment that is commonly used for
455 filter samples. In this study, these two sampling methods, cyclonic aerosol sampler and filter
456 sampler, showed different sampling efficiency. The additional treatment that is commonly used
457 for filter samples, such as recovering the sample from the filter, is one of the main differences
458 between these two methods. Besides, the air samples were collected directly into the running
459 buffer by cyclonic aerosol sampler while the other method collected samples on filters. The
460 characteristics of different type of sampling method might contribute to this different sampling
461 efficiency. We can assume that the cyclonic aerosol sampler is the better choice for
462 microorganism collection, and this assumption needs further confirmation.
463
464 In this study, bacterial *16S rDNA* and fungal *ITS* region sequencing were used to perform the
465 biological analysis of bioaerosols. *16S rDNA* sequencing is the determination of *16S rDNA*
466 segments in the microbial genome[30]. *16S rDNA* widely exists in prokaryotes with high
467 conservation and specificity, which makes it useful for the identification of microbial species[31].
468 Whole-genome sequencing requires only the extraction of genomic DNA and subsequent
469 sequencing. In addition to producing a large amount of data, this process also allows for a more
470 comprehensive analysis of microbial community structure. Besides, metagenomics can also be
471 used in this field of study by providing more information in the future. Handelsman *et al.* first
472 proposed the concept of the metagenome in a 1998 paper on microbes in soil[32]. In subsequent
473 studies, the concept of the metagenome was gradually accepted, and much research was
474 carried out on the microbes included in the human gut, ocean and soil[33-35]. With the support of
475 high-throughput sequencing technology, metagenomics has developed rapidly, and it plays an
476 increasingly important role in the study of pathogen detection. Traditional microbial research
477 methods mainly rely on culture for separation and purification. However, many studies cannot
478 be carried out because more than 99% of microbes cannot be cultured. In contrast to
479 traditional methods, metagenomics can take the genetic information of all the microbes in the
480 environment as a whole without the need to separate individual organisms[36]. A comprehensive
481 analysis of all the resulting microorganisms can be conducted directly.
482
483 In summary, this study showed several detection, sampling and analysis methods that could be
484 used for studies on the biological composition of environmental PM, including PM monitoring;
485 PM sampling by an Andersen six-stage sampler, a high-volume air sampler with filters or
486 cyclonic aerosol sampler; and subsequent biological analysis based on DNA sequencing. In
487 practice, these methods can be used under different environmental conditions, such as many
488 types of livestock farms. Our protocols and results may help other researchers all over the

489 world further explore the health impacts of fungal and bacterial bioaerosols in the
490 environment.
491

497 **DISCLOSURES:**
498 The authors have nothing to disclose.
499

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Figure 1
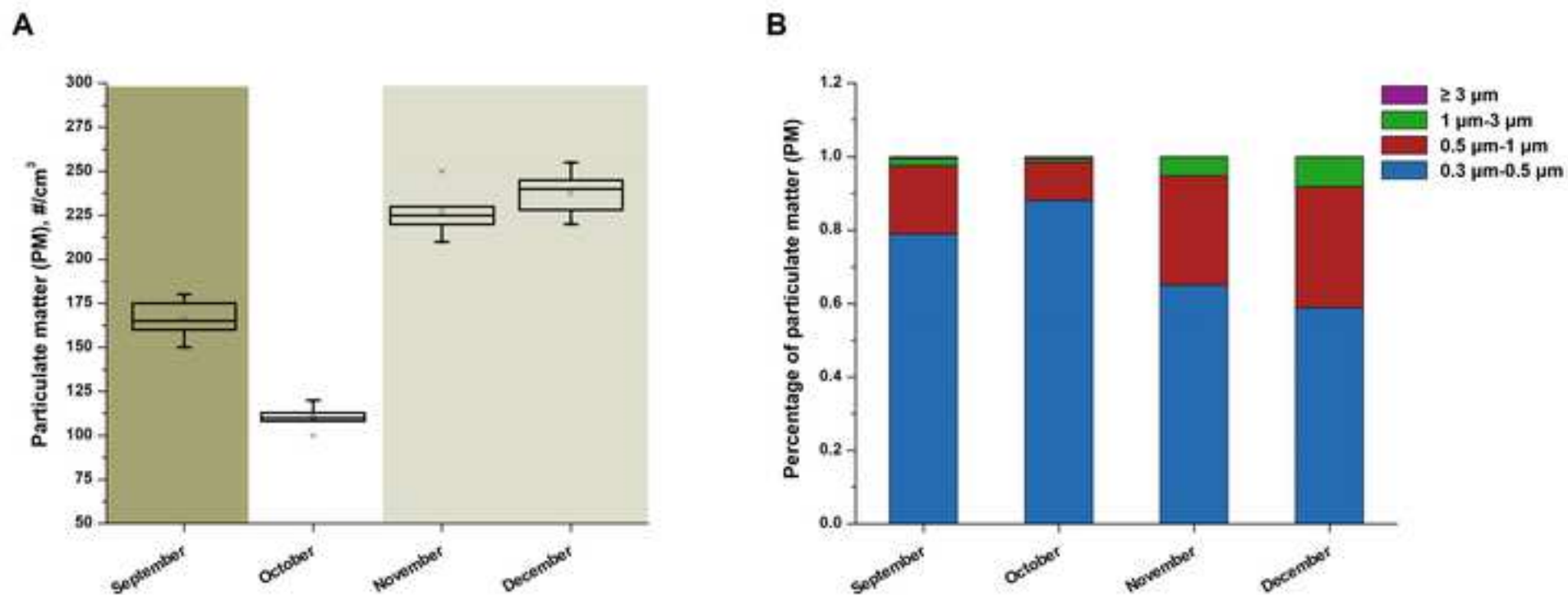
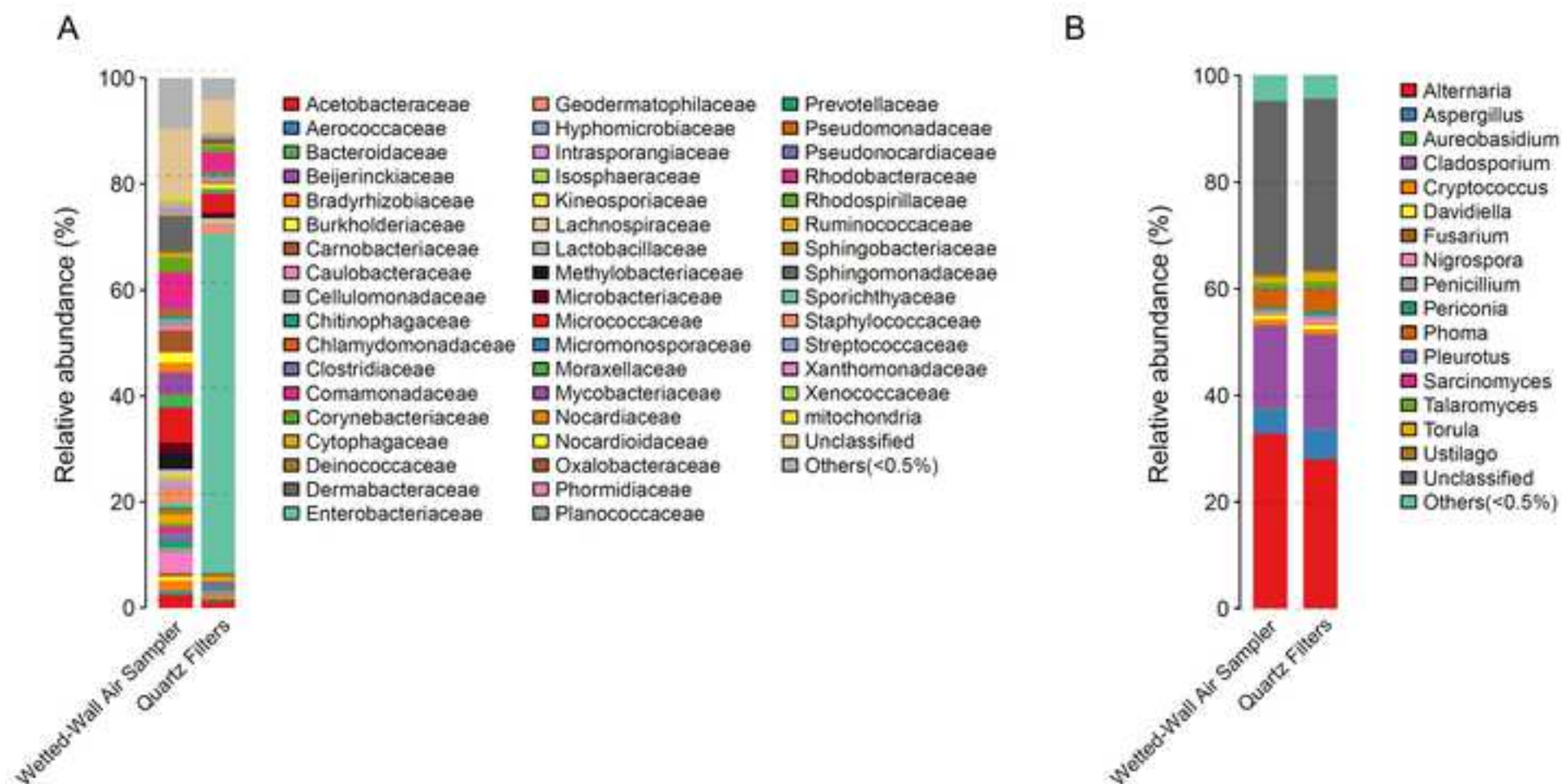Click here to access/download;Figure;Figure 1.JPG

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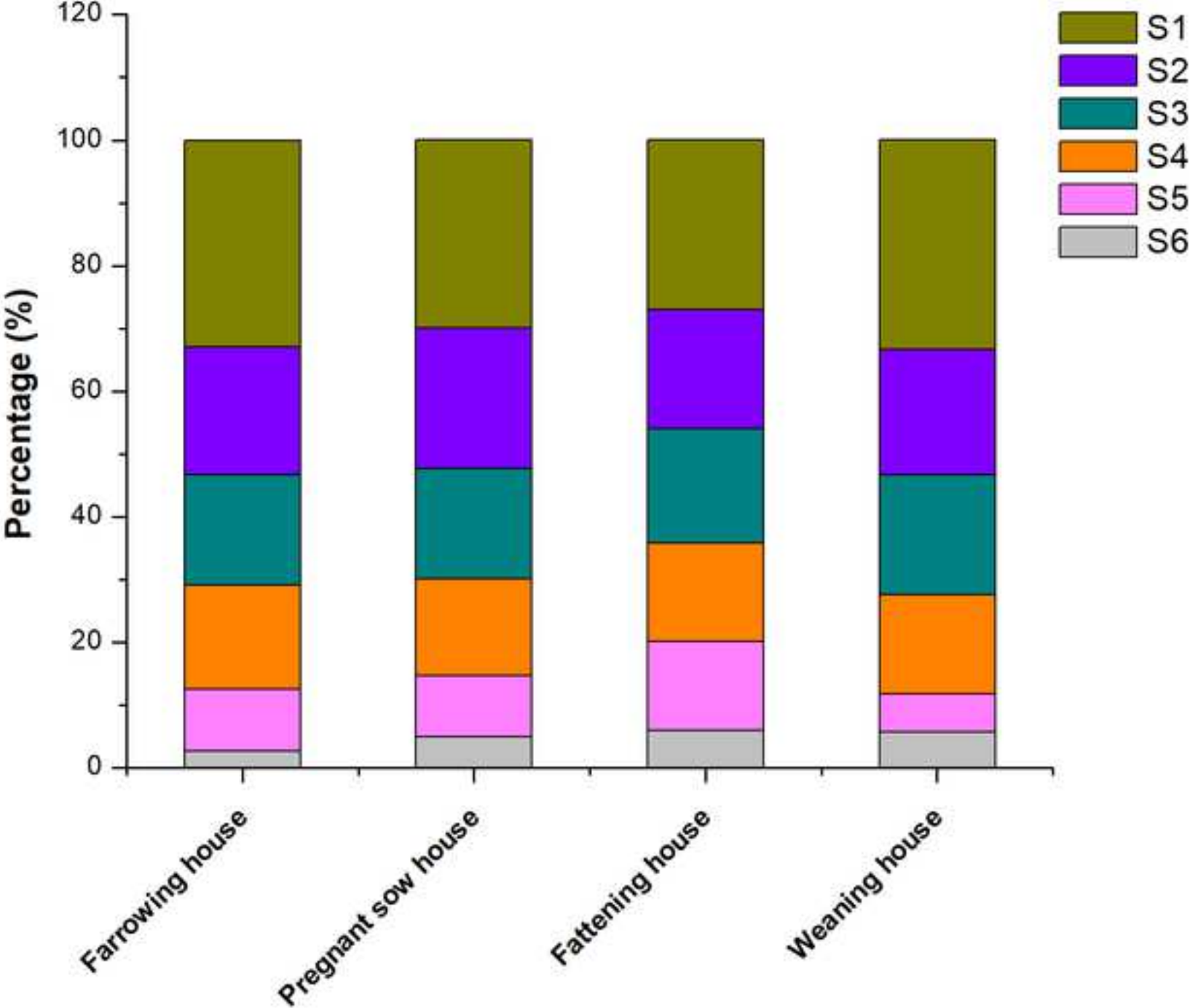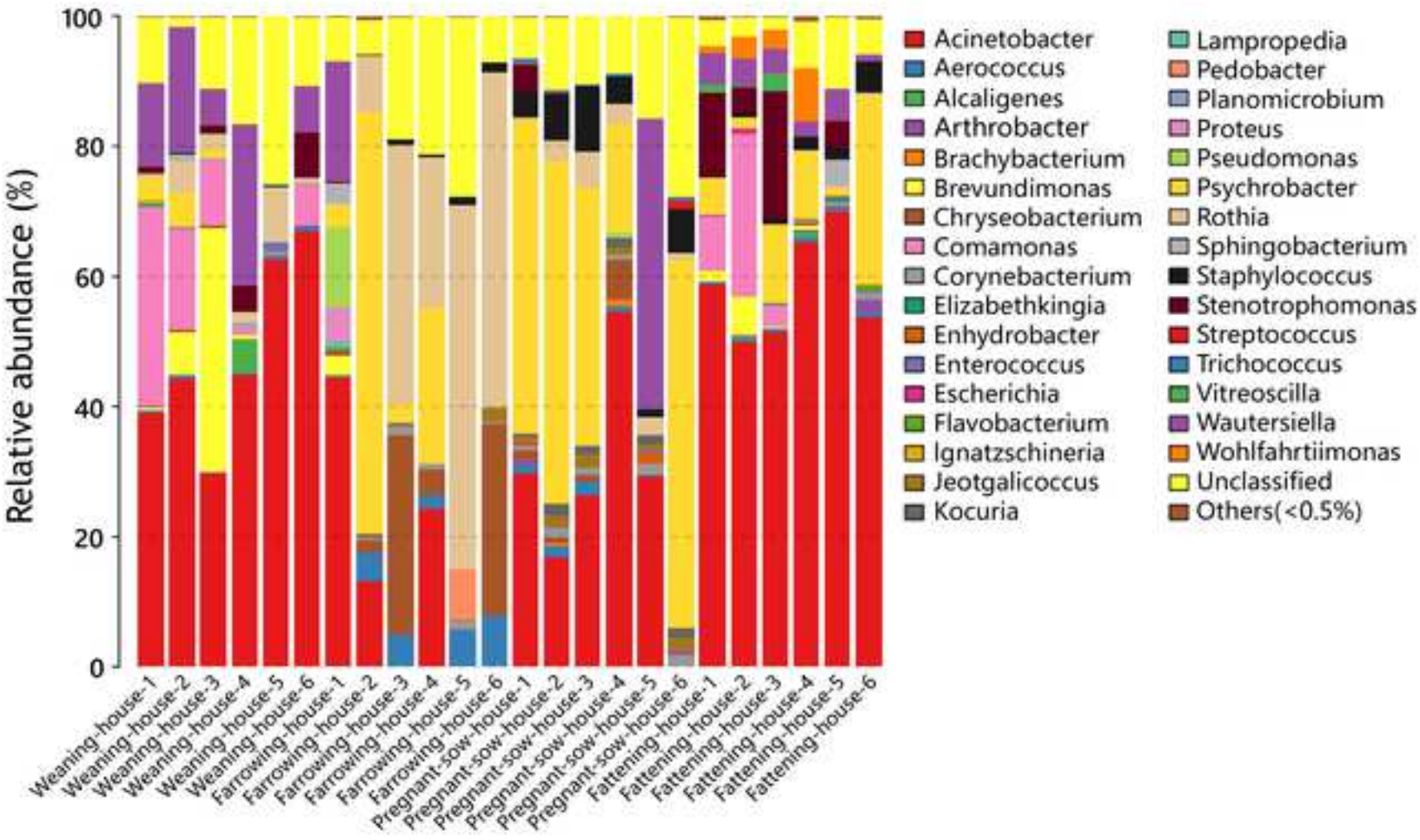Click here to access/download;Figure;Figure 2.JPG

Figure 3   Click here to access/download;Figure;Figure 3.jpg

Table 1. The environmental factors from September to December

Month	Temperature (°C)	Humidity (% rh)	Wind Speed (m/s)
September	22.9±0.9	64.1±2.4	0.06±0.005
October	12.1±0.7	38.6±1.7	0.05±0.005
November	4.3±0.4	62.2±3.9	0.02±0.004
December	1.6±0.1	67.8±2.3	0.02±0.003

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
airborne laser particle counter	TSI Inc, MN, USA	model 9306	
Andersen six-stage sampler	Tisch Inc, USA	TE-20-600	
AxyPrep multisource DNA Miniprep Kit	Axygen, NY, USA	AP-MN-MIS-GDNA-50G	
FastPfu Polymerase	TransGen Inc., Beijing, China	AP221-01	
High-volume air sampler	Beijing HuaRui HeAn Technology Co., Ltd., China	HH02-LS120	
Real-Time PCR System	Thermo Fisher Scientific, USA	Applied Biosystems® 7500	
Soybean-Casein Digest Agar	Becton, Dickinson and company, MD, USA	211043	
Tissuquartz filters	Pall, NY, USA	7204	
Wetted-Wall Air Sampler	Research International, Inc. 17161 Beaton Road SE Monroe, Washington 98272-1034 USA	SASS 2300	

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612542.6   For questions, please contact us at submissions@jove.com or +1.617.945.9051.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response 2: Thank you for your suggestion. We are sure that there are no spelling or grammar issues.

2. Please do not highlight notes for filming.
Response 2: Thank you for your suggestion. We have revised the highlighted part including no note.

3. Please do not abbreviate journal titles for all references.
Response 3: Thank you for your suggestion. All the journal titles have been corrected.

4. Step 1.1.2: How to measure and record? Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Response 4: Thank you for your good question. More details were added to the step 1.1.2 as follow:

   1.1.2) In total, measure and record 6 different particle size classes (0.3-0.5 μm, 0.5-0.7 μm, 0.7-2.5 μm, 2.5-5 μm, 5-10 μm and > 10 μm) simultaneously every 5 min. Also measure 4 other particle size classes (0.3-0.5 μm, 0.5-1 μm, 1-3 μm and ≥ 3 μm). Collect the air samples though the sampling hole on the top of the sampler and use the test modules inside the sampler to measure the particle size of each PM. Then the data was stored automatically. All the processes above can be performed automatically after the relative parameters, including sampling time and counting range, were set though the mini touching displayer of the airborne laser particle counter.

5. 4.2.1: What's the centrifugation rate (in x g) and time?
Response 5: Thank you for your good question. Centrifuge the bacteria sample at 2000 x g for 5 min.

6. 4.2.5: What's the centrifugation rate (in x g) and time?
Response 6: Thank you for your good question. After brief centrifugation at 1000 x g for 30 s (no residues on the wall), put the centrifugal tube in 56 °C water for 10 min.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript has been substantially improved by providing appropriate modification and other information to each comment.

I recommend that the revised manuscript may be accepted without modifications.

Response: Thank you very much for your comments and suggestions. We are looking forward to the publication of this manuscript.

Reviewer #2:

Manuscript Summary:

This manuscript has revised in dependence on the comments of editor and reviewers. However, there are some remaining issues.

I recommend publication after major revision.

Major Concerns:

1) The section of Materials and Methods is thought to be fragmented and does not include sufficient information about sampling methods.

Response 1: Thank you for your good question. More details were added to the step 1.1.2 as follow:

1.1.2) In total, measure and record 6 different particle size classes (0.3-0.5 μm, 0.5-0.7 μm, 0.7-2.5 μm, 2.5-5 μm, 5-10 μm and > 10 μm) simultaneously every 5 min. Also measure 4 other particle size classes (0.3-0.5 μm, 0.5-1 μm, 1-3 μm and ≥ 3 μm). Collect the air samples though the sampling hole on the top of the sampler and use the test modules inside the sampler to measure the particle size of each PM. Then the data was stored automatically. All the processes above can be performed automatically after the relative parameters, including sampling time and counting range, were set though the mini touching displayer of the airborne laser particle counter.

2) The description style for data results (taxonomic composition) has not reached to research article level, and now just data values are shown in the result section.

Response 2: Thank you very much for your comments and suggestions. According to your suggestion, we have shortened the description of the taxonomic composition results as follow:

The air samples were collected in four different types of piggeries by using an Andersen six-stage sampler and then cultured under suitable conditions. The whole-genome DNA of the culturable bacteria collected from each particle stage was extracted and detected by bacterial *16S rDNA* and fungal *ITS* region sequencing. A total of 91 genera and 158 species of bacteria were identified in the culturable bacteria in piggeries. The culturable bacteria community structures in four different types of piggeries, including farrowing house, pregnant sow house, fattening house and weaning house are shown in **Figure 4** with data from stage I to stage VI. The content of different predominant bacterial genera is not the same among different piggeries.

3) The aims of study also should be re-written for showing the comparison

between liquid and filter sampling and between 16S and ITS.

Response 3: Thank you very much for your comments and suggestions. We have added the relative description as follow:

In this study, we explored multiple types of analyses of bioaerosols, including PM number monitoring, bioaerosol collection and biological composition analysis. Air samples were collected by a cyclonic aerosol sampler, a high-volume air sampler with filters and an Andersen six-stage sampler. Then, the samples collected by these three samplers were analysed by biological analysis including bacterial *16S DNA* and fungal *ITS* sequencing to determine their biological compositions. Herein, we show representative results from the bioaerosol samples collected during Beijing hazy days and from livestock farms indicating that bioaerosols might have great impacts on human and animal health. <u>The comparison between liquid and filter sampling methods were also explored in this study mainly based on the data from *16S DNA* and fungal *ITS* sequencing.</u>

Minor Concerns:
"16S rRNA genes" and "ITS" are not shown as italic letters.

Response: Thank you very much for your comments and suggestions. I have revised all the "16S rRNA" and "ITS", all of them are italic letters in the revised manuscript.