



Title	Usefulness of a 3D-printing air sampler for capturing live airborne bacteria and exploring the environmental factors that can influence bacterial dynamics
Author(s)	Mori, Saaya; Ishiguro, Sakura; Miyazaki, Satoru et al.
Citation	Research in Microbiology, 172(6), 103864 https://doi.org/10.1016/j.resmic.2021.103864
Issue Date	2021-09-01
Doc URL	https://hdl.handle.net/2115/86696
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Mori Ishiguro Miyazaki et al text 20210625 Revise.pdf



1 **Usefulness of a 3D-printing air sampler for capturing live airborne bacteria**
2 **and exploring the environmental factors that can influence bacterial**
3 **dynamics**

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42

43

44 **Abstract**

45

46 We created a handmade 3D-printed air sampler to effectively collect live airborne bacteria, and
47 determined which environmental factors influenced the bacteria. Bacterial colony forming
48 units (CFUs) in the air samples ($n=37$) were monitored by recording the environmental changes
49 occurring over time, then determining the presence/absence of correlations among such
50 changes. The bacterial CFUs changed sharply and were significantly correlated with the DNA
51 concentrations, indicating that the captured bacteria made up most of the airborne bacteria.
52 Spearman's rank correlation analysis revealed significant correlations between the bacterial
53 CFU values and some environmental factors (humidity, wind speed, insolation, and 24-h
54 rainfall). Similarly the significant associations of CFU with humidity and wind speed were also
55 found by multiple regression analysis with box-cox transformation. Among our panel of
56 airborne bacteria (952 strains), 70 strains were identified as soil-derived *Bacillus* via the
57 production of *Escherichia coli*- and *Staphylococcus aureus*-growth inhibiting antibiotics and
58 by 16S rDNA typing. Soil-derived protozoa were also isolated from the air samples. We
59 conclude that the airborne bacteria mainly derived from soil can alter in number according to
60 environmental changes. Our sampler, which was created by easy-to-customize 3D printing, is a
61 useful device for understanding the dynamics of live airborne bacteria.

62

63 *Keywords:* 3D printing, air sampler, air bacteria, protozoa, environmental factors

64 **1. Introduction**

65

66 Because some human pathogenic bacteria cause infectious diseases through airborne inhalation
67 [1], monitoring the population dynamics of these bacteria and their biological properties by live
68 capture is extremely important if we are to provide safe air in the public environment. It is also
69 true that many living things on the earth are inevitably connected through the air. Accumulating
70 studies have revealed that soil-derived bacteria can contribute to the human gut microbiome,
71 probably by entering the body through inhaled air [2]. Therefore, elucidating the factors that
72 can influence the dynamics of bacteria floating in the air, and controlling these dynamics for the
73 benefit of human health, is worthwhile. However, research in this area is very limited. One
74 reason for this is that commercially available devices for air collection are not well equipped to
75 efficiently recover live bacteria [3-6].

76 Sampling methods and equipment for collecting airborne bacteria include membrane
77 filtration, impinger, impaction, cyclonic separation, and electrostatic precipitation [3-6] with
78 some interesting application examples in such farms or hospitals [7-10]. On the other hand, it is
79 also true that there are the following limitations. It is difficult to efficiently collect live airborne
80 bacteria using commercially available membrane filtration-based air samplers because the
81 filters in them can dry out during use [11]. Another method, which is considered an excellent
82 tool for recovering live bacteria, uses an impinger device where the sample is collected into
83 liquid; however, the amount of intake air is too small to be useful and the collected liquid can
84 evaporate, making it unsuitable for long sampling periods [12]. Other commercial air samplers
85 suffer from poor airflow design and resultant poor bacterial recovery [13]. Although it is
86 necessary to collect a large amount of air without damaging the sampled bacteria, no ideal air

87 sampler currently exists; hence, the need to develop more efficient devices for capturing live
88 airborne bacteria exists.

89 With the ability to design devices without any shape restrictions, 3D printing technology has
90 come to the fore as a way of creating devices for biomedical science and other scientific fields
91 [14]. Therefore, we used 3D printing technology to create an air sampler for efficiently
92 collecting live bacteria in the air, and explored the environmental factors that can affect
93 bacterial prevalence. Here, we show that the airborne bacteria mainly derived from soil can
94 change dramatically in number depending on environmental factors, and that our sampler
95 created with easy-to-customize 3D printing is a useful device for gaining information on the
96 dynamics of airborne bacteria.

97

98

99 **2. Materials and Methods**

100

101 *2.1. 3D printing and materials*

102 A device consisting of several parts was designed by SketchUp software (Trimble Inc.,
103 Sunnyvale, CA, USA). An important feature is its smooth air flow operation, which was
104 secured by installing an air inlet and four outlets with a 12-cm diameter PC fan as the main
105 engine for air collection (Fig. 1A). Next, the parts were individually printed on 3D printer Da
106 Vinci Jr. 2.0 Mix (XYZPrinting, New Taipei City, Taiwan) and then assembled (Fig. 1B and C).
107 A biomass plastic, called poly-lactic acid, was used as the construction material. After capture,
108 air particles were collected on agar plates or dishes filled with sterile water (see below). While
109 the device can be connected to the household power supply, it can also be driven by a USB

110 battery making it portable. In addition, the blueprints (stl files) of this air sampler (registered
111 name: “Air Sampler”) have been uploaded on the Thingiverse (<https://www.thingiverse.com/>),
112 which is a popular site for publishing original files for 3D-printing, to make it available to
113 everyone.

114

115 *2.2. Monitored factors*

116 Environmental factors [atmospheric pressure (hPa), temperature (°C), humidity (%), wind
117 speed (m/sec), insolation (MJ/m²), 24-h rainfall (mm)] at the meteorological observation point
118 (north latitude: 43.06048; east longitude: 141.32917) closest to the sampling site (within 2.12
119 km) were obtained from the Japan Meteorological Agency
120 (<http://www.jma.go.jp/jma/index.html>).

121

122 *2.3. Air sampling room*

123 Air sampling was performed in an air sampling room (1.2 m×2.25 m×2.47 m) on the third floor
124 of our faculty building to avoid direct contamination by soil particles. The distance was 8.25 m
125 from the ground (north latitude: 43,07470, east longitude: 141.34567). The window used for
126 outside air intake (0.55 m×1.4 m) was covered by a net to prevent insect entry, and was opposite
127 to the entrance door (0.47 m×1.8 m) to exhaust the air and both were kept opened so that the
128 clean air from outside could flow into the room efficiently.

129

130 *2.4. Preliminary experiment to confirm the air sampler's performance*

131 To determine whether airborne bacteria would fall onto agar plates placed in the device's four
132 canisters in the same way, air samples were taken 11 times from 15 February to 31 April, 2020

133 in the air sampling room. Four R2A-agar plates (BD, Franklin Lakes, NJ, USA) were placed in
134 each canister in the air sampler, and the air was sampled for 2 h. After collection, the R2A-agar
135 plates were cultured at 30°C for 5 days, and the number of colonies on each of the four plates
136 was counted and the total bacterial numbers were used to assess the recovery rate differences
137 among the canisters. Concurrently, bacteria in the air sampling room that made contact with the
138 four plates were sampled as a control.

139

140 2.5. Sample collection

141 Air samples were taken 37 times (from 6 April–13 August 2020) in the air sampling room. In
142 one sampling, four R2A-agar plates (used for measuring viable bacterial counts and creating an
143 environmental bacteria panel) or four glass dishes containing 25 mL of sterilized water (for
144 protozoa isolation and DNA extraction) were individually placed in each canister of the air
145 sampler, and the air was collected for 2 h. The plates were then cultured under aerobic
146 conditions at 30°C for 2 days, and the number of colonies on the four plates were summed as
147 total bacterial numbers. In addition, 36 colonies were randomly selected from each plate and
148 panelized on R2A-agar plates to identify the soil-derived *Bacillus* strains that produce
149 antibiotics against *Escherichia coli* and *Staphylococcus aureus* [15, 16] (see below). All the
150 water collected from the four glass petri dishes was transferred to two 50 mL tubes, placed in a
151 filtration device (Advantec Toyo, Toyo, Japan) on a clean bench, and then filtered through a
152 0.22 µm filter. The filter was directly used for protozoa isolation, and stored at –20° C for DNA
153 extraction (see below). In addition, air samples were also taken 7 times (2h each) (from 21
154 April-11 May 2021) in the air sampling room with both our handmade air sampler and a filter
155 sampler used in our previous study [17], and then the number of colonies on R2A-agar plate

156 were compared.

157

158 2.6. DNA extraction and DNA concentration measurements

159 Total DNA was extracted using the Power Soil DNA Isolation kit (MO BIO Laboratories, Inc.,
160 Carlsbad, CA, USA). First, each filter was cut into small pieces, and the pieces were placed in
161 the bead-containing tube included in the kit, followed by heating at 65°C for 10 min and stirring
162 for 2 min. The DNA was then extracted according to the manufacturer's protocol. Because the
163 amount of DNA in the sample was too low to be measured directly, the concentration of
164 bacterial DNA in the samples was indirectly determined by comparing the Cq value from
165 quantitative PCR (qPCR) targeting of the 16S rDNA in the DNA extracted with a known
166 number of *E. coli* DH5 α cells. The qPCR was performed by CFX Connect (BioRad, Hercules,
167 CA, USA) with KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and the following primers:
168 forward: 5'-TCC TAC GGG AGG CAG CAG T-3'; reverse: 5'-GGA CTA CCA GGG TAT CTA
169 ATC CTG TT-3') [18].

170

171 2.7. Identification of soil-derived *Bacillus*

172 Altogether, 952 colonies were panelized from the air samples. The colonies were spotted onto
173 an R2A-agar plate smeared with *E. coli* (ATCC25922) and *S. aureus* (ATCC29213), and then
174 cultured at 30°C for 5 days and assessed for their ability to inhibit the growth of *E. coli* and/or *S.*
175 *aureus*. Bacteria showing growth inhibition were selected as possible *Bacillus* candidates.
176 Finally, whether they were *Bacillus* or not was determined by 16S rDNA sequencing of
177 amplicons using 27F: 5'-AGA GTT TGA TCM TGG CTC AG-3' (forward primer) and 1492R:
178 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (reverse primer) [19].

179

180 *2.8. Isolation of protozoa (amoebae and ciliates)*

181 As mentioned above, after air trapping, the filters were used to isolate protozoa. The filters were
182 cultured at 30°C by adding 4 mL of Page's amoeba saline (PAS) [20] with 3 grains of brown
183 rice for one week on 6-well plates. Among the cultured samples, 50 µL of a sample with the
184 appearance of amoebae was dropped onto the center of a non-nutrient agar (NNA) plate on
185 which heat-inactivated *E. coli* (from a stock collection in our laboratory) were spread as a food
186 source. The plates were cultured at 30°C and the amoebae (trophozoites and cysts) were
187 morphologically identified by the presence or absence of migration out from the dropped spot
188 on the NNA plate. Also, 1 mL of each sample with a ciliate-like appearance was combined with
189 9 mL of PAS. After centrifugation at 600 × g or 5 min, a further 9 ml of PAS solution was added,
190 the mixture was recentrifuged at 600 × g for 5 min, and then left standing upwards at room
191 temperature. After 2 h, 2 ml of the solution from the surface of each tube was carefully
192 withdrawn, centrifuged at 600 × g for 5 min, and the pellet was continuously cultured in
193 Sonneborn's Paramecium Medium (SPM) [21] for ciliate culturing. For samples with
194 significantly increased ciliate numbers, DNA was extracted from 100 µL of the culture solution
195 using the Cica genus DNA extraction kit (Kanto Chemical Co., Inc., Tokyo, Japan). 18S rDNA
196 from the ciliates was PCR-amplified using P-SSU-342f 5'-CTT TCG ATG GTA GTG TAT
197 TGG ACT AC-3' (forward) and Medlin B 5'-TGA TCC TTC TGC AGG TTC ACC TAC-3'
198 [22] (reverse) primers.

199

200 *2.9. Direct sequencing and database accession numbers*

201 The PCR-amplified products were separated by agarose gel electrophoresis and extracted from

202 the gels using the FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan)
203 according to the manufacturer's protocol, and then sequenced by Fasmac (Kanagawa, Japan).
204 Ciliate types were confirmed by BLASTn interrogation of the nucleotide sequence database
205 (National Center Biotechnology Information BLASTn: [https://blast.ncbi.nlm.nih.gov/Blast](https://blast.ncbi.nlm.nih.gov/Blast.cgi).
206 [cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)). The accession numbers of the nucleotide sequences used for the BLASTn analysis are
207 listed in Table S1. The sequences from this study have been deposited in the DNA Data Bank of
208 Japan (DDBJ, <https://www.ddbj.nig.ac.jp/index.html>).

209

210 *2.10. Statistical analysis*

211 Comparisons between groups were performed using Mann-Whitney's U test. *P*-values of less
212 than 0.05 were considered statistically significant. The presence of a correlation for the total
213 CFU and an environmental factor or DNA concentration was determined by Spearman's rank
214 correlation test. A correlation coefficient value of >0.3 or <-0.3 with a *p*-value of less than 0.05
215 was considered significant. Calculations were performed in Excel for Mac (2011) with
216 Statcel3C.

217 Furthermore, to verify normality of the data, Shapiro-Wilk test was conducted. If *P*-value is
218 ≥ 0.05 , factors with such data were chosen as explanatory variables in multiple regression
219 analysis of criterion variable. Meanwhile, if *p*-value in Shapiro-Wilk test is less than 0.05, the
220 data were further transformed by box-cox transformation, which can transform it to normal
221 distribution [23]. The transformed data were tested by Shapiro-Wilk test again. The data which
222 normality can be assumed (*p*-value in Shapiro-Wilk test ≥ 0.05) were also chosen as
223 explanatory variables in multiple regression analysis of criterion variable. Finally, the
224 significance of each explanatory variable in regression model against criterion variable was

225 tested by *t*-test, and a *p*-value of less than 0.05 was considered significant. In addition,
226 normality test and multiple regression analysis with box-cox transformation were conducted by
227 the statistical software R ver 3.6.3.

228

229

230 **3. Results**

231

232 *3.1. Performance and effectiveness of the newly developed air sampler*

233

234 Because the air volume of the fan used for our air sampler was 69.11 CFM (cubic feet per
235 minute) (35.31 CFM=1,000 L/min), the airflow rate was theoretically reached at approximately
236 2,000 L/min, indicating that our sampler, as compared with others, can produce extremely high
237 airflows [filter sampler (40 L/min), impinger (12.5 L/min)] [17, 24]. Next, because the sampler
238 is equipped with four canisters, we examined whether the number of bacteria captured in each
239 canister differed. As a result, although the total bacterial colony numbers changed daily, the
240 number of bacteria among the canisters was significantly correlated (correlation values:
241 0.66272–0.85262; *p* vales: 0.02627–0.00183) (Fig. S1A), which confirms the accuracy of
242 3D-printing. Furthermore, the total bacterial count [average (SD): 687 (494)] was significantly
243 higher [average (SD): 249 (224)] in the plate left in the room (*p*=0.0143) (Fig. S1B). In addition,
244 we also compared the bacterial collection efficiency (bacterial count per m³) between our air
245 sampler and a filter sampler used in previous study [17], showing the efficiency of the air
246 sampler [average (SD): 2.0707 (1.713)] was higher than those of the filter sampler [average
247 (SD): 0.4116 (0.4174)] (*p*=0.0088) (Fig. S2). Thus, the air sampler that we created appears to

248 exhibit excellent performance when compared to those of the filter sampler, which is
249 commonly used, indicating that the devise may be superior to others that are available for
250 exploring the dynamics of live airborne bacteria.

251

252 *3.2. Correlation between air bacterial dynamics and environmental factors*

253 First, air samples for measuring viable bacterial counts and DNA amounts were taken 37 times
254 from 6 April–13 August 2020 in the air sampling room. Although the value of each sample
255 changed sharply depending on the collection day (Fig. 2A and B), both values were
256 significantly correlated ($r=0.334$, $p=0.041$) (Fig. 2C). These results indicate that the number of
257 bacteria in a sample can change greatly in response to the influence of an environmental factor,
258 and the culturable bacteria under aerobic conditions represented most bacteria floating in the
259 air.

260 We next monitored several environmental factors including atmospheric pressure (hPa),
261 temperature (°C), humidity (%), wind speed (m/sec), insolation (MJ/m²), and 24-h rainfall
262 (mm) during the sampling period. Unlike the other environmental factors, from early spring to
263 summer the temperature gradually rose to 26°C on the final sampling day (13 August 2020),
264 indicating the monitoring accuracy of the environmental factors (Fig. S3). We also conducted
265 normality test, however, indicating that atmospheric pressure and humidity only followed
266 normal distribution (Table S2, See the factors with the value of ≥ 0.05 in “ p -value in normality
267 test”). Thus, since normality cannot be assumed for most data, Spearman’s rank correlation
268 coefficients, which is a non-parametric analysis, were calculated. As a result, significant
269 correlations between the bacterial CFU values and some environmental factors were found
270 [$r=-0.518$ ($p=0.001$) (humidity), $r=0.416$ ($p=0.012$) (wind speed), $r=0.464$ ($p=0.005$)

271 (insolation), and $r=-0.334$ ($p=0.044$) (24-h rainfall)] (Fig. 3). Although temperature was
272 weakly correlated ($r=0.242$), no significant difference was found ($p=0.145$).

273 Furthermore, the data lacking normality were simply transformed to normal distribution
274 by a box-cox method [23], and some factors (CFU, temperature, wind speed) could be
275 successfully converted to data showing a normal distribution (Table S2, See the factors with the
276 value of ≥ 0.05 in “ p -value in normality test after box-cox transformation”). Therefore,
277 multiple regression analysis was performed with the CFU as an objective variable, using the
278 factors (atmospheric pressure, temperature, humidity, and wind speed) as explanatory variables
279 for which the normal distribution was found in two time verifications. As a result, similar as
280 correlation analysis by Spearman’s rank correlation coefficient, the values of multiple
281 regression coefficient showed significant negative correlation between humidity and CFU
282 ($p=0.02$, t -test in multiple regression analysis) and significant positive correlation between
283 wind speed and CFU ($p= 0.03$, t -test in multiple regression analysis) (Table S3).

284 Thus, as expected, the number of airborne bacteria was negatively or positively affected by
285 environmental factors such as humidity, wind speed, insolation, and 24-h rainfall.

286

287 3.3. Search for the origins of the air-floating microbes

288 Many of the microorganisms floating in the air are expected to have risen from the soil. To
289 confirm this hypothesis, soil-derived *Bacillus* was detected by its ability to produce antibiotics
290 that can inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*, and 16S rDNA
291 typing was used to type our panel of airborne bacteria (952 strains). As a result, 70 strains were
292 identified as soil-derived *Bacillus* members, most of which were *Bacillus subtilis* (15 strains,
293 21%) (Table S1 and Fig. 4).

294 Soil-derived protozoa (amoebae and ciliates) were concurrently isolated from 33.3% of the
295 air samples (Fig. S4A). In continuous culture, typical cysts were seen on the surfaces of plates
296 with crawling amoebae (Fig. 5A), and large numbers of vacuole-forming ciliates were observed
297 (Fig. 5B). Despite the lack of sufficient amoebal growth in liquid cultures to allow DNA
298 sequence identification of any amoeba species, we did identify two ciliates (*Colpoda lucida* and
299 *C. inflata*) that universally inhabit soils [25] (Table S1 and Fig. S4A). We also assessed whether
300 the prevalence of protozoa was correlated with any of the studied environmental factors, but no
301 correlations were found (Fig. S4B) probably because a small number were tested.

302 Thus, the live airborne bacteria we captured were mainly derived from soil and varied
303 depending on the environmental factors prevailing at the time of sampling.

304

305

306 **4. Discussion**

307

308 Using an air sampler created with easy-to-customize 3D printing technology [14], we have
309 shown that the airborne bacteria mainly derived from soil can change dramatically in number
310 depending on the environmental factors prevalent at the sampling time. Specifically, we found
311 that the number of bacteria floating in the air decreased when the humidity rose and increased
312 when drying progressed, but further evidence will be needed to confirm this. This is the first
313 application of 3D printing used to obtain an air sampler for assessing environmental conditions
314 related to bacterial dynamics in the air over time.

315 There are three reasons for using the 3D printing approach for an air sampler. First, despite
316 the advantages of commercially available air samplers [3-6], few can efficiently collect large

317 numbers of live bacteria from the air because their air flow pathways are not well designed [13].
318 Second, commercially available products have fixed air-flow shapes [26], so it is not possible to
319 capture live bacteria and collect DNA for metagenomic analysis using same devise. Third, the
320 biggest reason, is that commercial products are extremely costly [27].

321 Therefore, our air sampler customized by 3D printing was designed with the following
322 features. It costs only approximately 20,000 yen (150 EUR, 182 USD, 129 GBP) for the
323 materials needed to create a device with a secure air flow path. By selecting a capture tool in the
324 sampler, it can be used for various applications such as capturing live bacteria or extracting
325 DNA for metagenomic analysis. It is also possible to easily create a replica from the blueprint,
326 and sampling various locations concurrently is also possible. Because this sampler can be easily
327 assembled and disassembled without the use of special equipment, it can be carried wherever
328 researchers want to sample. In addition, as mentioned above in the results section, the printing
329 is accurate such that the number of bacteria captured in the four canisters is uniform with no
330 large variations. Meanwhile, differences between the number of colonies on the plates from the
331 sampling room were seen, as expected. Thus, the device could work on collecting airborne
332 bacteria efficiently.

333 We found that viable bacterial counts were significantly correlated with DNA concentrations
334 ($r=0.334$, $p=0.041$) (Fig. 2C), indicating that our sampler efficiently recovered live bacteria
335 from the air. However, the correlation coefficient was not very high. The amount of DNA in
336 each sample was determined as a relative value by qPCR targeting of 16S rDNA because the
337 DNA amount per sample was small. Therefore, it seems that some small errors in DNA
338 amplification were inevitable and resulted in some sample variation [28]. Thus, we believe that
339 the data secured by simple culturing under aerobic conditions captured most of the bacteria in

340 the air.

341 As expected, we found significant correlations between the number of live bacteria captured
342 by our device and each of the four environmental factors [humidity ($r=-0.518$, $p=0.001$), wind
343 speed ($r=0.416$, $p=0.012$), insolation ($r=0.464$, $p=0.005$), and 24-h rainfall ($r=-0.334$,
344 $p=0.044$)] (Fig. 3). This indicates that while wind speed and insolation can positively affect the
345 number of airborne bacteria, humidity and 24-h rainfall can negatively affect the bacterial
346 numbers. Because water is essential for all living microorganisms, the presence of water or a
347 humid environment is necessary for their survival and growth [29]. Although humid conditions
348 can cause soils to become compacted and the microorganisms in them less able to disperse
349 upwards [30, 31], a humidity-guaranteed state is considered to be advantageous for their
350 survival, making their movement less advantageous to them. In contrast with humid conditions,
351 dryness, which is a detrimental situation for microorganisms [30, 31], may force them to
352 become airborne on the wind and on updrafts. On the other hand, although multiple regression
353 analysis with the conversion to normal distribution supported the results of Spearman's rank
354 correlation analysis, there were some factors (insolation and 24-h rainfall) that could not be
355 converted to a normal distribution even when the box-cox conversion was used, suggesting that
356 some environmental factors cannot be simply applied to linear regression models. Hence, in
357 order to find more accurate associations, further study for developing new regression model
358 will be required for such environmental factors.

359 Accumulative evidence indicates that among environmental factors, temperature can affect
360 the survival of airborne infections agents (bacteria, viruses, and fungi) and, in particular,
361 increasing temperatures are intricately intertwined with other environmental factors such as
362 humidity or sunlight, which can inactivate these agents [32-35]. Our previous study found a

363 positive correlation between walker occupancy and airborne bacteria [17]. This study also
364 found that airborne bacterial numbers increased in line with increases in temperature and
365 humidity in the presence of small airborne particles in the Sapporo underground pedestrian
366 space [17]. However, in the present study, temperature was uncorrelated with the number of air
367 bacteria [$r=0.242$ (Spearman's rank correlation test), $p=0.07$ (t -test in multiple regression
368 analysis)] (Fig. 3D, and Table S2). The exact reason for this contradiction needs to be clarified,
369 but temperature is obviously a factor that can indirectly affect other environmental factors such
370 as humidity and air updrafts, and it is possible that such complex situations led to these results.
371 Further study will be needed to clarify this.

372 In the present study, *Bacillus* spp., some of which produce antibiotics, were used as an index
373 for soil bacteria. Of the 952 paneled strains, 78 formed an inhibition circle when tested against
374 *E. coli* or *S. aureus* and, as expected, 89% of the bacteria forming these circles were identified
375 as *Bacillus* by 16S rDNA ribotyping. With *Bacillus* accounting for 7% of the panelized strains,
376 we concluded that the main source of airborne bacteria is soil. Pertinently, recent studies have
377 highlighted the probiotic potential of environmentally-derived *Bacillus*, which produces
378 various antibiotics [36-38]. It is unclear what antibiotic types are produced by the strains we
379 identified as *Bacillus* in the present study, but the air sampler that we created may be a very
380 effective device for exploring *Bacillus* strains as probiotics.

381 Surprisingly, we also found that ciliates and amoebae are transported in the air. Although we
382 could not identify the amoebal species, the ciliate species was *Colpoda*, a protozoan that mainly
383 inhabits the soil [25]. This result indicates that large microorganisms are also floating in the air
384 along with soil-derived bacteria. It is well established that such protozoa provide a place for the
385 growth and survival of human pathogenic bacteria such as *Legionella* or *Mycobacterium* in

386 natural environments [39, 40]. That cooling water, hot springs, humidifiers, and outdoor
387 temperature control units can be simultaneously contaminated with microorganisms that move
388 through the air supports the assertion that these pathogenic bacteria can survive in harsh
389 environments. Therefore, controlling protozoal movement in the air could reduce the risk of
390 infection by protozoa-related human pathogenic bacteria.

391 We conclude that the dynamics of the airborne bacteria mainly derived from soil can
392 significantly change depending on certain environmental factors. In particular, we found that
393 although the number of airborne bacteria can reduce depending on humidity and rainfall, their
394 numbers can rapidly increase with strengthening wind speeds and/or sunlight. We also found
395 that some protozoa can be floating in the air along with airborne bacteria, presumably
396 supporting the survival of human pathogenic bacteria in harsh environments. Thus, these
397 findings provide novel insights into how to properly control the risk of infection from
398 environmentally-derived human pathogenic bacteria. Moreover, our sampler created with
399 easy-to-customize 3D printing is a useful device for understanding live airborne bacterial
400 dynamics.

401

402

403 **Conflict of interest**

404 The authors declare no conflicts of interest associated with this manuscript.

405

406

407 **Acknowledgments**

408 This study was supported in part by Grants-in-Aid for Scientific Research (KAKEN) (grant

409 number: 20K20613). We thank Sandra Cheesman, PhD, from Edanz Group
410 (<https://en-author-services.edanz.com/ac>) for editing a draft of this manuscript.

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415 **Supporting Information**

416

417 **Table S1.** List of accession numbers for the gene sequences registered in DDBJ.

418

419 **Table S2.** Normality of each factor verified by Shapiro-Wilk test with or without box-cox
420 transformation.

421

422 **Table S3.** Multiple regression coefficient of each factor with normal distribution against CFU.

423

424 **Figure S1.** Homogeneity of airborne bacterial capture on the plates installed in four canisters
425 (A), and comparison of the number of airborne bacteria recovered with or without the sampler
426 (B).

427

428 **Figure S2.** Comparison between the bacterial collection efficiency (bacterial count per m³)
429 between our air sampler and a filter sampler used for our previous study. Air samples were also
430 taken 7 times (2h each) (from 21 April-11 May 2021) in the air sampling room with both our
431 handmade air sampler and a filter sampler used in our previous study [15], and the number of
432 colonies on R2A-agar plate were compared (See the Materials and Methods). *, a *p*-value with
433 a statistical significant.

434

435 **Figure S3.** Seasonal variation in the studied environmental factors. A, atmospheric pressure
436 (hPa). B, temperature (°C). C, humidity (%). D, wind speed (m/sec). E, insolation (MJ/m²). F,
437 24-h rainfall (mm).

438

439 **Figure S4.** Protozoa (amoebae and ciliates) frequency in the collected air samples (A), and their

440 correlation with environmental factors (B). Volume (m³), amount of air collected in each
441 sampling. ns, not significant. Negative, samples where the appearance of protozoa was absent.
442 Positive, samples with the appearance of protozoa.

443

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445 **References**

- 446 1. Wall DH, Nielsen UN, Six J. Soil biodiversity and human health. *Nature* 2015;528:69-76.
- 447 2. Blum WEH, Zechmeister-Boltenstern S, Keiblinger KM. Does Soil Contribute to the
448 Human Gut Microbiome? *Microorganisms* 2019;7:287.
- 449 3. Haddrell AE, Thomas RJ. Aerobiology: Experimental Considerations, Observations, and
450 Future Tools. *Appl Environ Microbiol* 2017;83:e00809-17.
- 451 4. Dybwad M, Skogan G, Blatny JM. Comparative testing and evaluation of nine different air
452 samplers: end-to-end sampling efficiencies as specific performance measurements for
453 bioaerosols applications. *Aero Sci Technol* 2015;48:282-295.
- 454 5. Zhen H, Han T, Fennell DE, Mainelis G. A systemic comparison of four bioaerosols
455 generators: affect on culturability and cell membrane integrity when aerosolizing
456 *Escherichia coli* bacteria. *J Aero Sci* 2014;70:67-79.
- 457 6. Xu Z, Wei K, Wu Y, Shen F, Chen Q, Li M, Yao M. Enhancing bioaerosol sampling by
458 Andersen impactors using mineral-oil-spread agar plate. *PLoS One* 2013;8:e56896.
- 459 7. Cabo Verde S, Almeida S, Matos J, Guerreiro D, Meneses M, Faria, T, Botelho D, Santos M,
460 Viegas C. Microbiological assessment of indoor air quality at different hospital sites. *Res*
461 *Microbiol* 2015;166:557-563.
- 462 8. Keessen E, Donswijk C, Hol S, Hermanus C, Kuijper E, Lipman L. Aerial dissemination of
463 *Clostridium difficile* on a pig farm and its environment. *Environ Res* 2011;111:1027-1032.
- 464 9. Marchand G, Duchaine C, Lavoie J, Veillette M, Cloutier Y. Bacteria emitted in ambient air
465 during bronchoscopy - a risk to health care workers? *Am J Infect Control*
466 2016;44:1634-1638.
- 467 10. Siller P, Daehre K, Rosen K, Münch S, Bartel A, Funk R, Nübel U, Amon T, Roesler U.

- 468 Low airborne tenacity and spread of ESBL - /AmpC - producing *Escherichia coli* from
469 fertilized soil by wind erosion. Environ Microbiol 2021, doi:10.1111/1462-2920.15437
470 (Online ahead of print).
- 471 11. Mbareche H, Veillette M, Bilodeau GJ, Duchaine C. Bioaerosol Sampler Choice Should
472 Consider Efficiency and Ability of Samplers To Cover Microbial Diversity. Appl Environ
473 Microbiol 2018;84:e01589-18.
- 474 12. Haig CW, Mackay WG, Walker JT, Williams C. Bioaerosol sampling: sampling
475 mechanisms, bioefficiency and field studies. J Hosp Infect 2016;93:242-55.
- 476 13. Lin C, Solera Garcia MA, Timmis R, Jones KC. Design and laboratory testing of a new
477 flow-through directional passive air sampler for ambient particulate matter. J Environ
478 Monit 2011;13:753-761.
- 479 14. Liaw CY, Guvendiren M. Current and emerging applications of 3D printing in medicine.
480 Biofabrication. 2017;9:024102.
- 481 15. Zhao P, Xue Y, Gao W, Li J, Zu X, Fu D, Bai X, Zuo Y, Hu Z, Zhang F. *Bacillaceae*-derived
482 peptide antibiotics since 2000. Peptides. 2018;101:10-16.
- 483 16. Olishavska S, Nickzad A, Déziel E. *Bacillus* and *Paenibacillus* secreted polyketides and
484 peptides involved in controlling human and plant pathogens. Appl Microbiol Biotechnol.
485 2019;103:1189-1215.
- 486 17. Okubo T, Osaki T, Nozaki E, Uemura A, Sakai K, Matsushita M, Matsuo J, Nakamura S,
487 Kamiya S, Yamaguchi H. Walker occupancy has an impact on changing airborne bacterial
488 communities in an underground pedestrian space, as small-dust particles increased with
489 raising both temperature and humidity. PLoS One. 2017;12:e0184980.
- 490 18. Zucol F, Ammann RA, Berger C, Aebi C, Altwegg M, Niggli FK, Nadal D. Real-time

- 491 quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by
492 sequencing for species identification. J Clin Microbiol. 2006;44:2750-2759.
- 493 19. Hou Q, Bai X, Li W, Gao X, Zhang F, Sun Z, Zhang H. Design of Primers for Evaluation of
494 Lactic Acid Bacteria Populations in Complex Biological Samples. Front Microbiol.
495 2018;9:2045.
- 496 20. Page, FC. A New Key to Freshwater and Soil Gymnamoebae (ISBN-10:18711105021).
497 Freshwater Biological Association, Ambleside, UK (1988), 122 pp.
- 498 21. Rotterová J, Bourland W, Čepička I. Tropidoatractidae fam. nov., a Deep Branching
499 Lineage of Metopida (*Armophorea*, *Ciliophora*) Found in Diverse Habitats and Possessing
500 Prokaryotic Symbionts. Protist. 2018;169:362-405.
- 501 22. Leng J, Zhong X, Zhu RJ, Yang SL, Gou X, Mao HM. Assessment of protozoa in Yunnan
502 Yellow cattle rumen based on the 18S rRNA sequences. Mol Biol Rep. 2011;38:577-585.
- 503 23. Liquet B, Riou J. Correction of the significance level when attempting multiple
504 transformations of an explanatory variable in generalized linear models. BMC Med Res
505 Methodol 2013;13:75.
- 506 24. Lin X, Willeke K, Grinshpun S. Effect of Sampling Time on the Collection Efficiency of
507 All-Glass Impingers. AIHAJ. 1997;58:480-488.
- 508 25. W. Foissner. *Colpodea (Ciliophora)*. Protozoenfauna, vol. 4/1, Gustav Fischer Verlag,
509 Stuttgart, 1993.
- 510 26. Xiao H, Hung H, Harner T, Lei YD, Johnston GW, Wania F. A flow-through sampler for
511 semivolatile organic compounds in air. Environ Sci Technol 2007;41:250-256.
- 512 27. McLean TD, Moore ME, Justus AL, Hudston JA, Barbé B. Dynamic Radioactive Source
513 for Evaluating and Demonstrating Time-dependent Performance of Continuous Air

- 514 Monitors. *Health Phys* 2016;111:442-450.
- 515 28. Rebrikov DV, Trofimov DIu. Real-time PCR: approaches to data analysis (a review). *Prikl*
516 *Biokhim Mikrobiol.* 2006;42:520-528.
- 517 29. Esbelin J, Santos T, Hébraud M. Desiccation: An environmental and food industry stress
518 that bacteria commonly face. *Food Microbiol.* 2018;69:82-88.
- 519 30. Evans SE, Bell-Dereske LP, Dougherty KM, Kittredge HA. Dispersal alters soil microbial
520 community response to drought. *Environ Microbiol.* 2020;22:905-916.
- 521 31. Joung YS, Ge Z, Buie CR. Bioaerosol generation by raindrops on soil. *Nat Commun.*
522 2017;8:14668.
- 523 32. Wolkoff P. Indoor air humidity, air quality, and health - An overview. *Int J Hyg Environ*
524 *Health.* 2018;221:376-390.
- 525 33. Tang JW. The effect of environmental parameters on the survival of airborne infectious
526 agents. *J R Soc Interface.* 2009;Suppl 6:S737-746.
- 527 34. Schinköthe J, Scheinemann HA, Diederich S, Freese H, Eschbaumer M, Teifke JP, Reiche S.
528 Airborne disinfection by dry fogging efficiently inactivates SARS-CoV-2, mycobacteria
529 and bacterial spores and shows the limits of commercial spore carriers for process control.
530 *Appl Environ Microbiol.* 2020:AEM.02019-20.
- 531 35. Fujiyoshi S, Tanaka D, Maruyama F. Transmission of Airborne Bacteria across Built
532 Environments and Its Measurement Standards: A Review. *Front Microbiol.* 2017;8:2336.
- 533 36. Jeżewska-Frąckowiak J, Seroczyńska K, Banaszczyk J, Jedrzejczak G, Żylicz-Stachula A,
534 Skowron PM. The promises and risks of probiotic *Bacillus* species. *Acta Biochim Pol.*
535 2018;65:509-519.
- 536 37. Lee NK, Kim WS, Paik HD. *Bacillus* strains as human probiotics: characterization, safety,

- 537 microbiome, and probiotic carrier. Food Sci Biotechnol. 2019;28:1297-1305.
- 538 38. Olmos J, Acosta M, Mendoza G, Pitones V. *Bacillus subtilis*, an ideal probiotic bacterium to
539 shrimp and fish aquaculture that increase feed digestibility, prevent microbial diseases, and
540 avoid water pollution. Arch Microbiol. 2020;202:427-435.
- 541 39. Borella P, Guerrieri E, Marchesi I, Bondi M, Messi P. Water ecology of *Legionella* and
542 protozoan: environmental and public health perspectives. Biotechnol Annu Rev.
543 2005;11:355-380.
- 544 40. Falkinham Iii JO. The biology of environmental mycobacteria. Environ Microbiol Rep.
545 2009;1:477-487.
- 546

547 **Figure legends**

548

549 **Fig. 1.** The 3D-printing air sampler internal structure and design drawings with how to work
550 actually in operation. The 3D-printing air sampler was constructed by hand by assembling the
551 distinct parts designed by SketchUp software. **A.** Internal structure of the air sampler. **B.** Parts
552 and the assembled image on the design drawing. **C.** Actual air sampler in operation.

553

554 **Fig. 2.** Correlation between viable bacterial counts and DNA concentrations in the air samples.
555 **A.** Changes in viable bacterial counts during the sampling period. **B.** Change in total DNA
556 amounts during the sampling period. The amounts are shown as relative concentrations
557 (Materials and Methods). **C.** Correlation between the measured viable bacterial counts and the
558 DNA concentrations. r , correlation coefficient. A correlation coefficient value of >0.3 or <-0.3
559 with a p -value of less than 0.05 was considered significant.

560

561 **Fig. 3.** Correlation between viable bacterial counts in the air samples and environmental factors.
562 **A,** atmospheric pressure (hPa). **B,** temperature (in °C). **C,** humidity (as a %). **D,** wind speed
563 (m/sec). **E,** insolation (MJ/m²). **F,** 24-hour rainfall (mm). r , correlation coefficient. A
564 correlation coefficient value of >0.3 or <-0.3 with a p -value of less than 0.05 was considered
565 significant.

566

567 **Fig. 4.** Frequency of soil-derived *Bacillus*, as detected by the activities of *E. coli*- and *S.*
568 *aureus*-growth inhibiting antibiotics, and 16S rDNA typing of a panel of airborne bacteria.
569 ($n=952$) **A.** Representative images showing growth inhibition in *E. coli* (left) and/or *S. aureus*

570 (right) on LB agar plates from bacteria-producing antibiotics. Arrowheads show the bacterial
571 inhibition of *E. coli* and /or *S. aureus*. **B.** Identification of *Bacillus* using 16S rDNA typing of
572 the bacterial strains with inhibition rings.

573

574 **Fig. 5.** Representative images showing the soil-derived protozoa identified from the air samples.

575 **A.** The image shows crawling amoebic trophozoites with cysts (stars). Magnification, ×400. **B.**

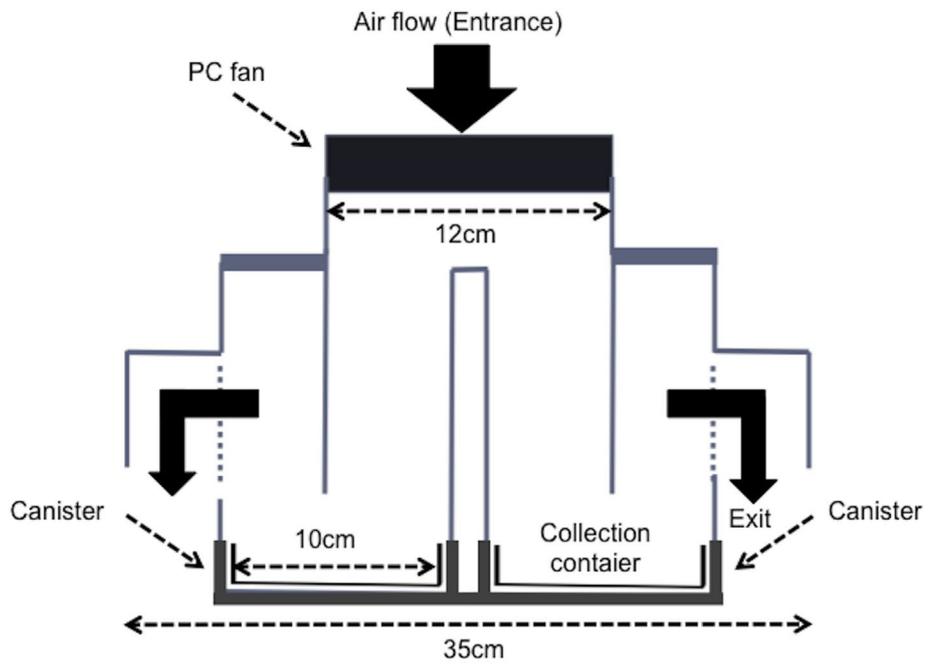
576 The image show ciliates with vacuoles (stars). Giemsa staining was performed. Magnification,

577 ×400.

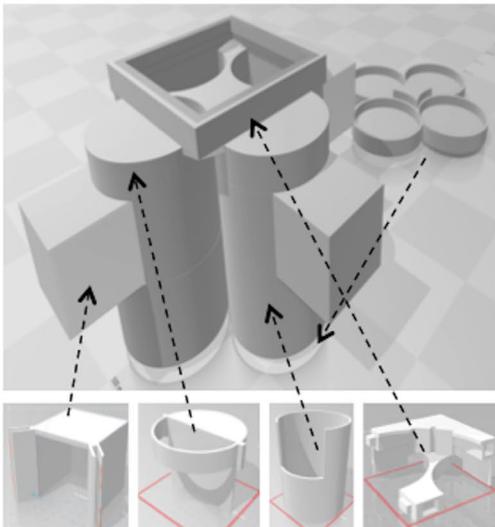
578

Figure 1

A



B



C



Figure 2

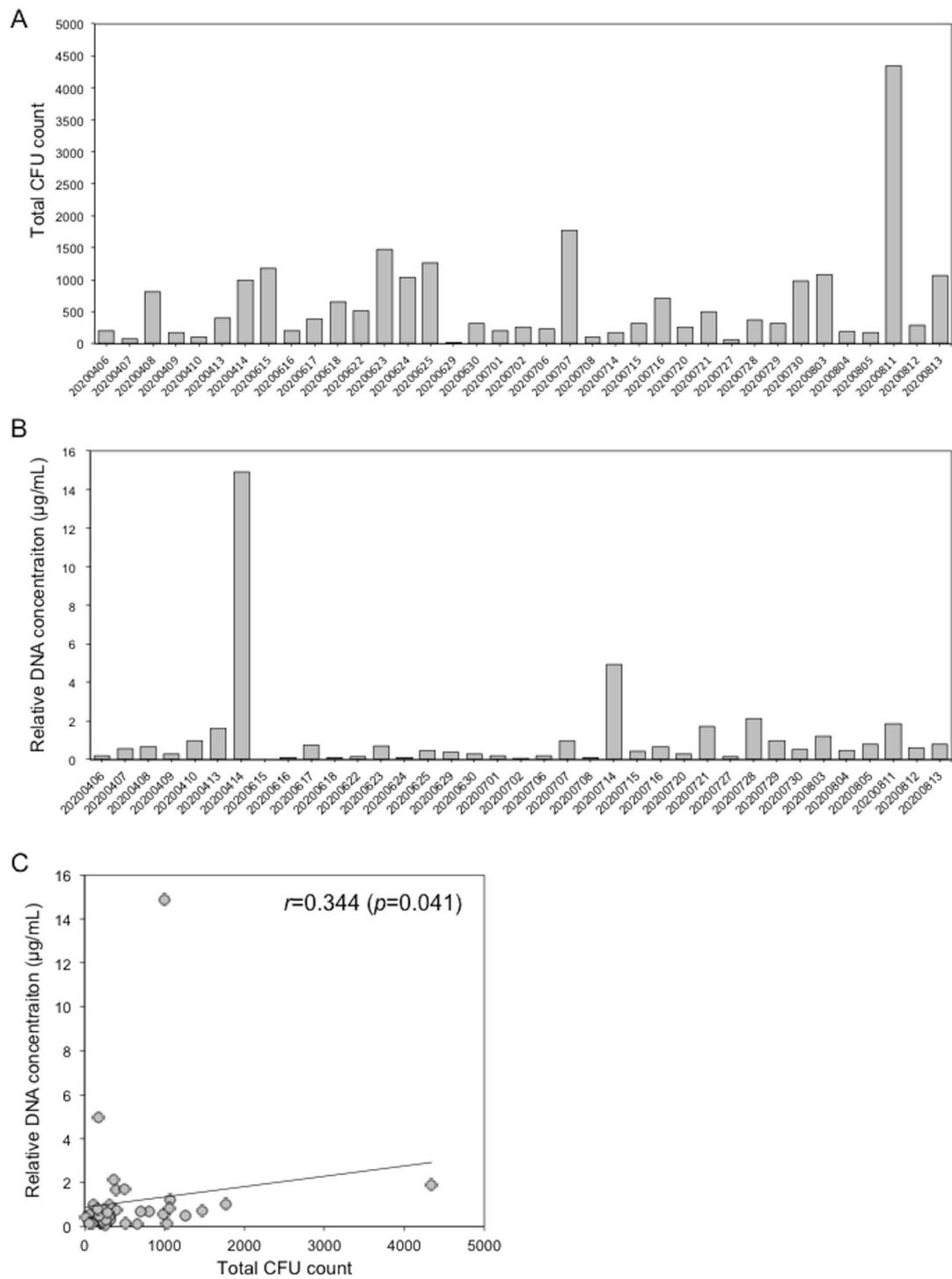


Figure 3

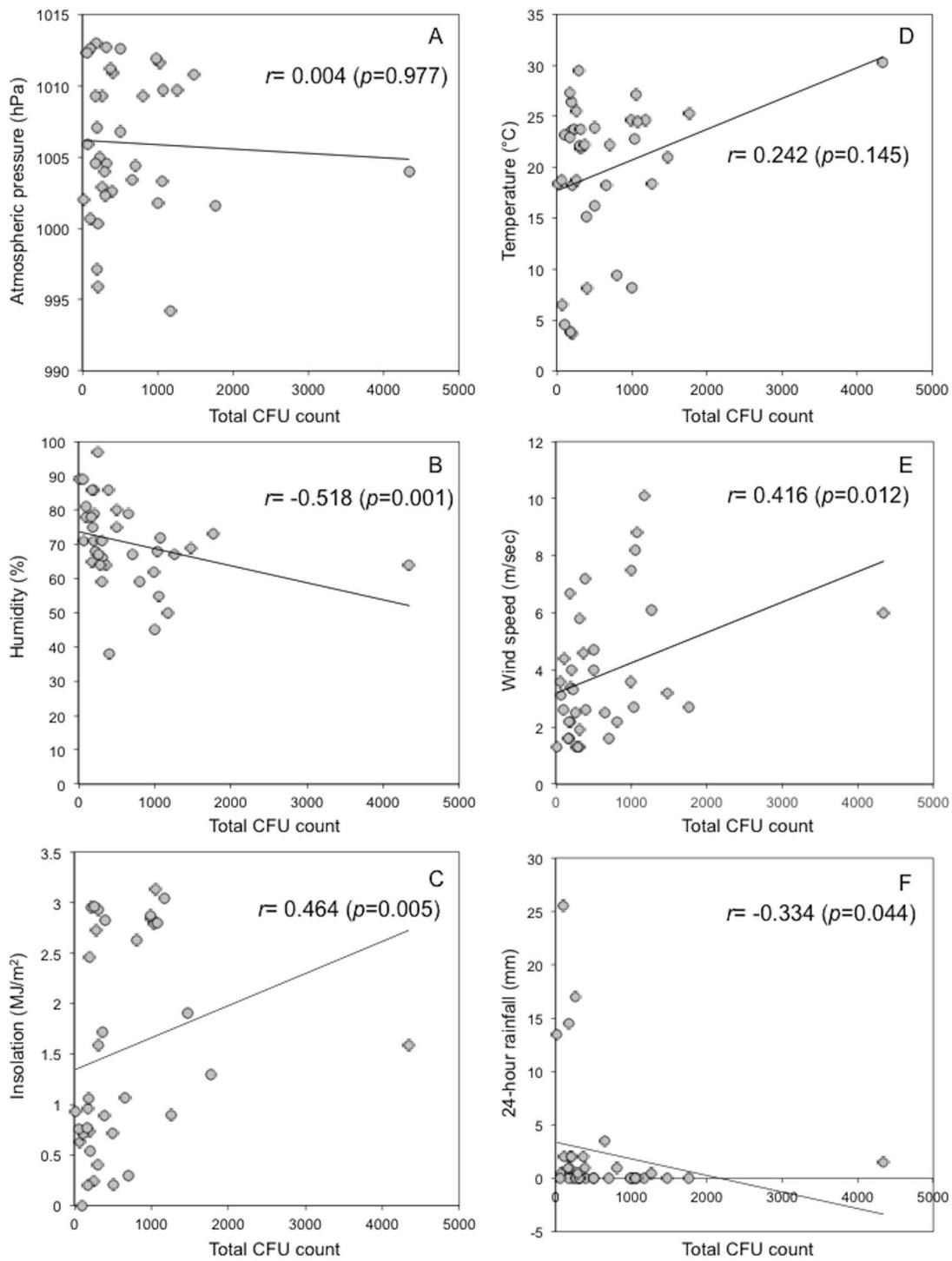
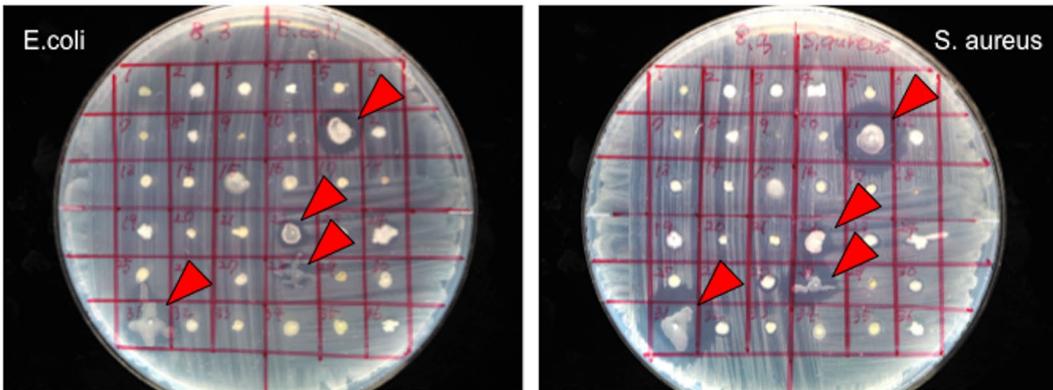


Figure 4

A



B

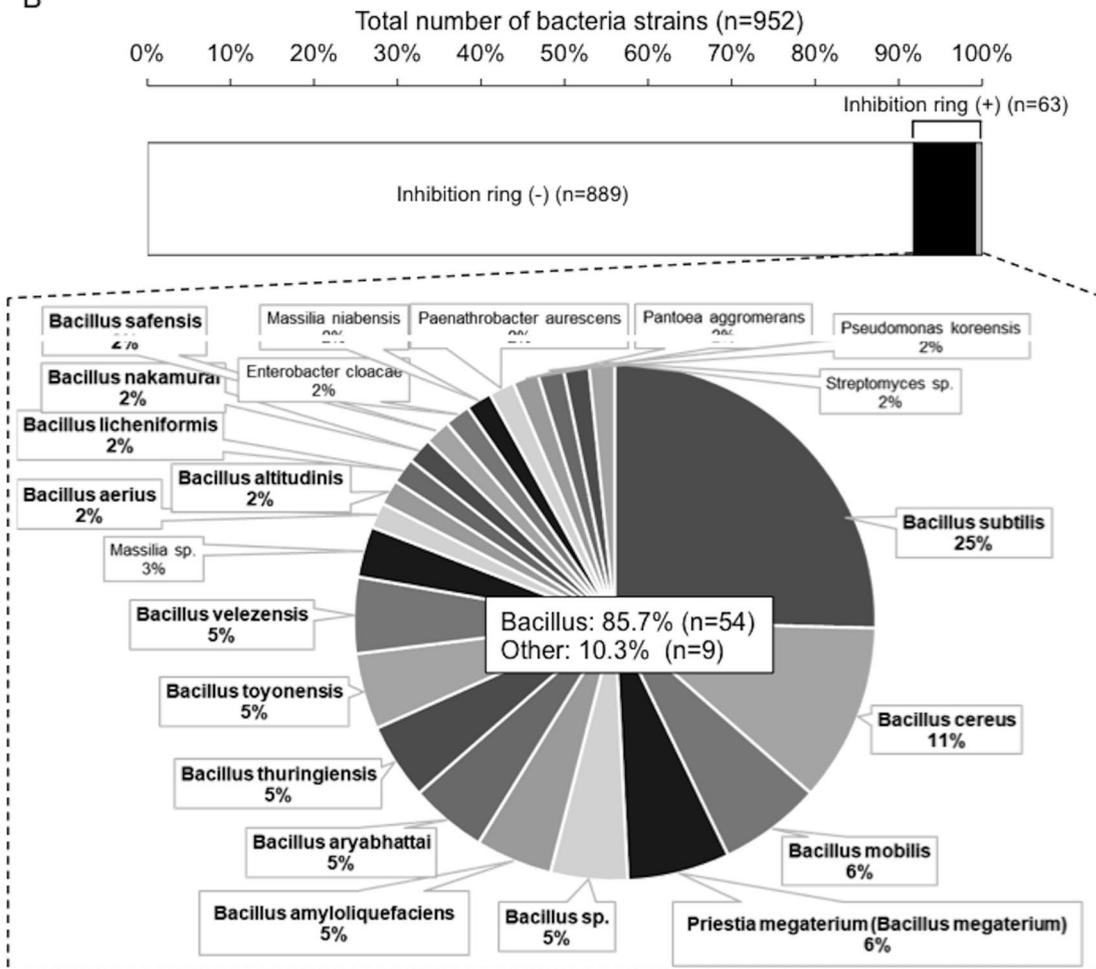
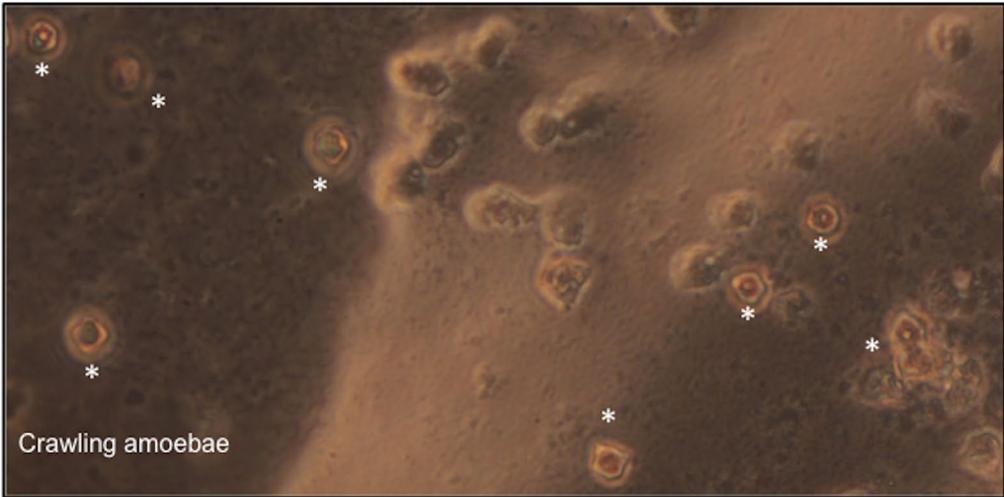


Figure 5

A



B

