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

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Abstract

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

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

1. Introduction

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

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

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

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

2. Materials and Methods

2.1. 3D printing and materials

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

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

2.2. Monitored factors

Environmental factors [atmospheric pressure (hPa), temperature (°C), humidity (%), wind speed (m/sec), insolation (MJ/m^2), 24-h rainfall (mm)] at the meteorological observation point (north latitude: 43.06048; east longitude: 141.32917) closest to the sampling site (within 2.12 km) were obtained from the Japan Meteorological Agency (http://www.jma.go.jp/jma/index.html).

2.3. Air sampling room

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

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

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

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

2.5. Sample collection

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

were compared.

2.6. DNA extraction and DNA concentration measurements

Total DNA was extracted using the Power Soil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). First, each filter was cut into small pieces, and the pieces were placed in the bead-containing tube included in the kit, followed by heating at 65°C for 10 min and stirring for 2 min. The DNA was then extracted according to the manufacturer's protocol. Because the amount of DNA in the sample was too low to be measured directly, the concentration of bacterial DNA in the samples was indirectly determined by comparing the Cq value from quantitative PCR (qPCR) targeting of the 16S rDNA in the DNA extracted with a known number of *E. coli* DH5α cells. The qPCR was performed by CFX Connect (BioRad, Hercules, CA, USA) with KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and the following primers: forward: 5'-TCC TAC GGG AGG CAG CAG T-3'; reverse: 5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3') [18].

2.7. Identification of soil-derived Bacillus

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

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

2.10. Statistical analysis

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

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

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

3. Results

3.1. Performance and effectiveness of the newly developed air sampler

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

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

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

4. Discussion

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

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

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

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

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

the air.

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

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

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

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

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

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

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

Conflict of interest

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

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Supporting Information

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

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

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

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

Figure S2. Comparison between the bacterial collection efficiency (bacterial count per m^3) between our air sampler and a filter sampler used for our previous study. Air samples were also taken 7 times (2h each) (from 21 April-11 May 2021) in the air sampling room with both our handmade air sampler and a filter sampler used in our previous study [15], and the number of colonies on R2A-agar plate were compared (See the Materials and Methods). *, a *p*-value with a statistical significant.

Figure S3. Seasonal variation in the studied environmental factors. A, atmospheric pressure (hPa). B, temperature (°C). C, humidity (%). D, wind speed (m/sec). E, insolation (MJ/m^2). F, 24-h rainfall (mm).

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

correlation with environmental factors (B). Volume (m^3), amount of air collected in each sampling. ns, not significant. Negative, samples where the appearance of protozoa was absent. Positive, samples with the appearance of protozoa.

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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/m^2). **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 *Bacillu*s, 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. coil* (left) and/or *S. aureus*

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

Fig. 5. Representative images showing the soil-derived protozoa identified from the air samples. **A**. The image shows crawling amoebic trophozoites with cysts (stars). Magnification, ×400. **B**. The image show ciliates with vacuoles (stars). Giemsa staining was performed. Magnification, ×400.

Figure 1

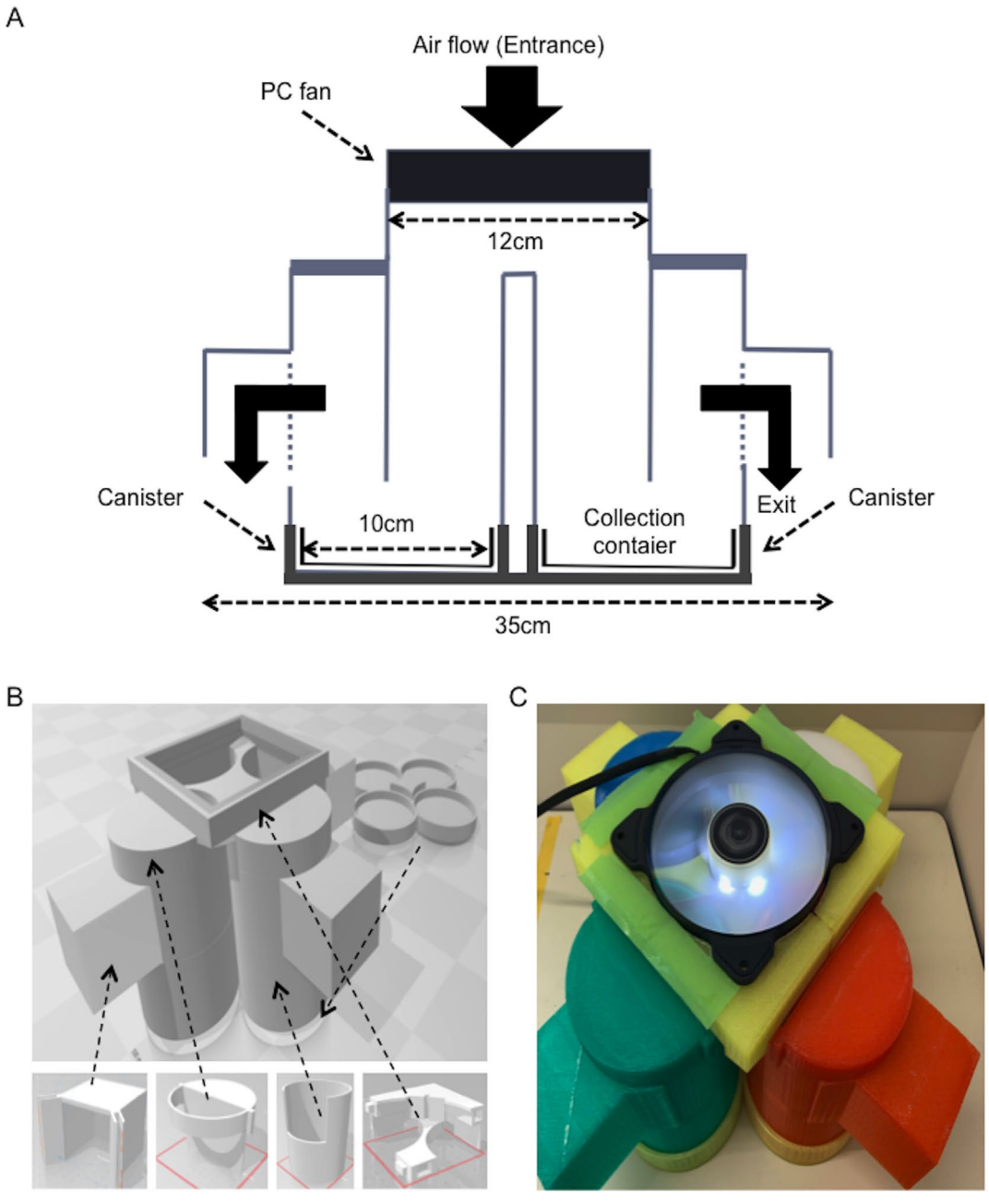

A
Air flow (Entrance)
PC fan
12cm
Canister
10cm
Collection
contaier
Exit
Canister
35cm
B
C

Figure 2

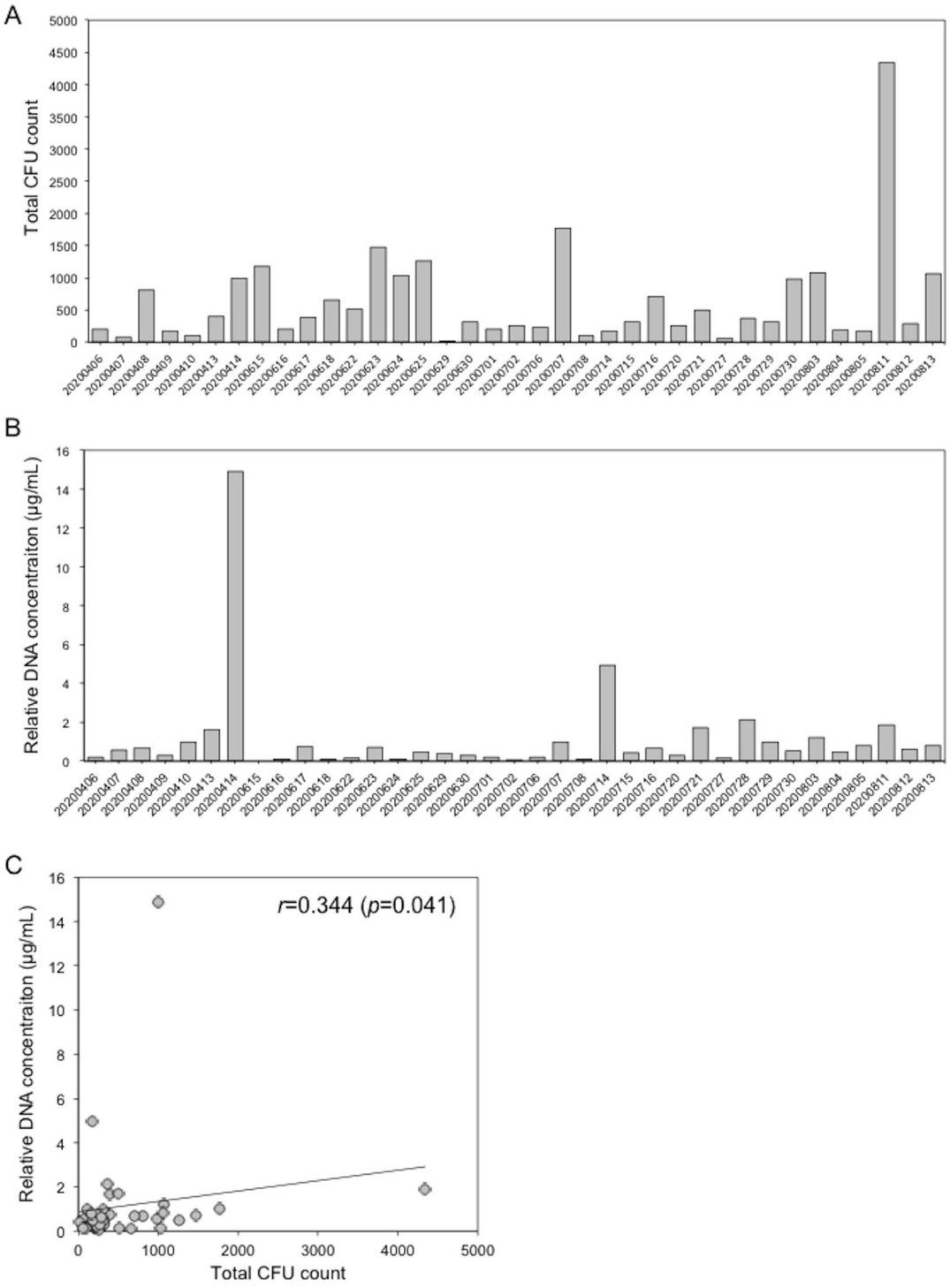

A
Total CFU count
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
20200406
20200407
20200408
20200409
20200410
20200413
20200414
20200615
20200616
20200617
20200618
20200622
20200623
20200624
20200625
20200629
20200630
20200701
20200702
20200706
20200707
20200708
20200714
20200715
20200716
20200720
20200721
20200727
20200728
20200729
20200730
20200803
20200804
20200805
20200811
20200812
20200813
B
Relative DNA concentraiton (µg/mL)
16
14
12
10
8
6
4
2
0
C
Relative DNA concentraiton (µg/mL)
r=0.344 (p=0.041)
16
14
12
10
8
6
4
2
0
0
1000
2000
3000
4000
5000
Total CFU count

Figure 3

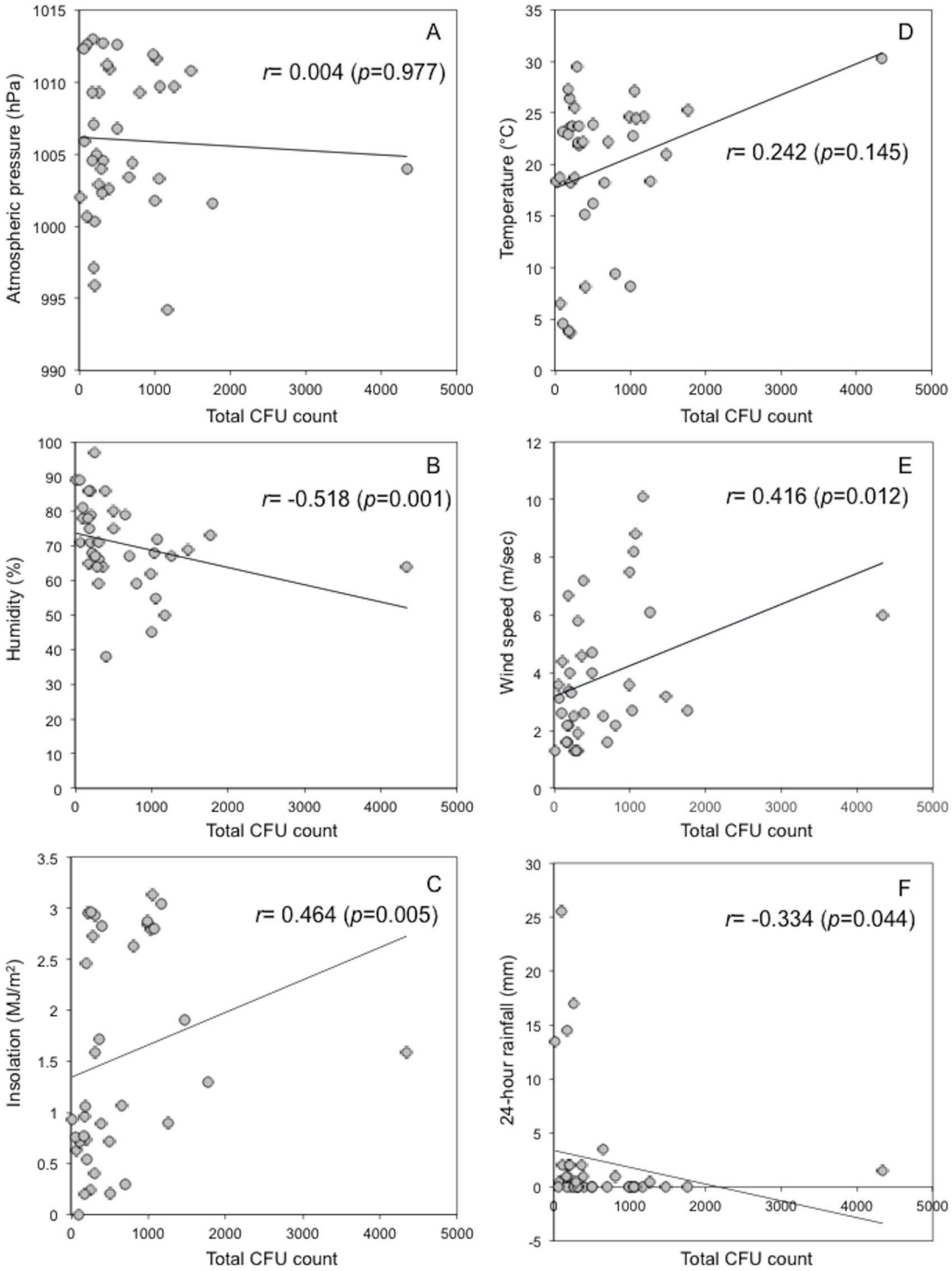

A
r= 0.004 (p=0.977)
Atmospheric pressure (hPa)
Total CFU count
D
r= 0.242 (p=0.145)
Temperature (°C)
Total CFU count
B
r= -0.518 (p=0.001)
Humidity (%)
Total CFU count
E
r= 0.416 (p=0.012)
Wind speed (m/sec)
Total CFU count
C
r= 0.464 (p=0.005)
Insolation (MJ/m²)
Total CFU count
F
r= -0.334 (p=0.044)
24-hour rainfall (mm)
Total CFU count

Figure 4

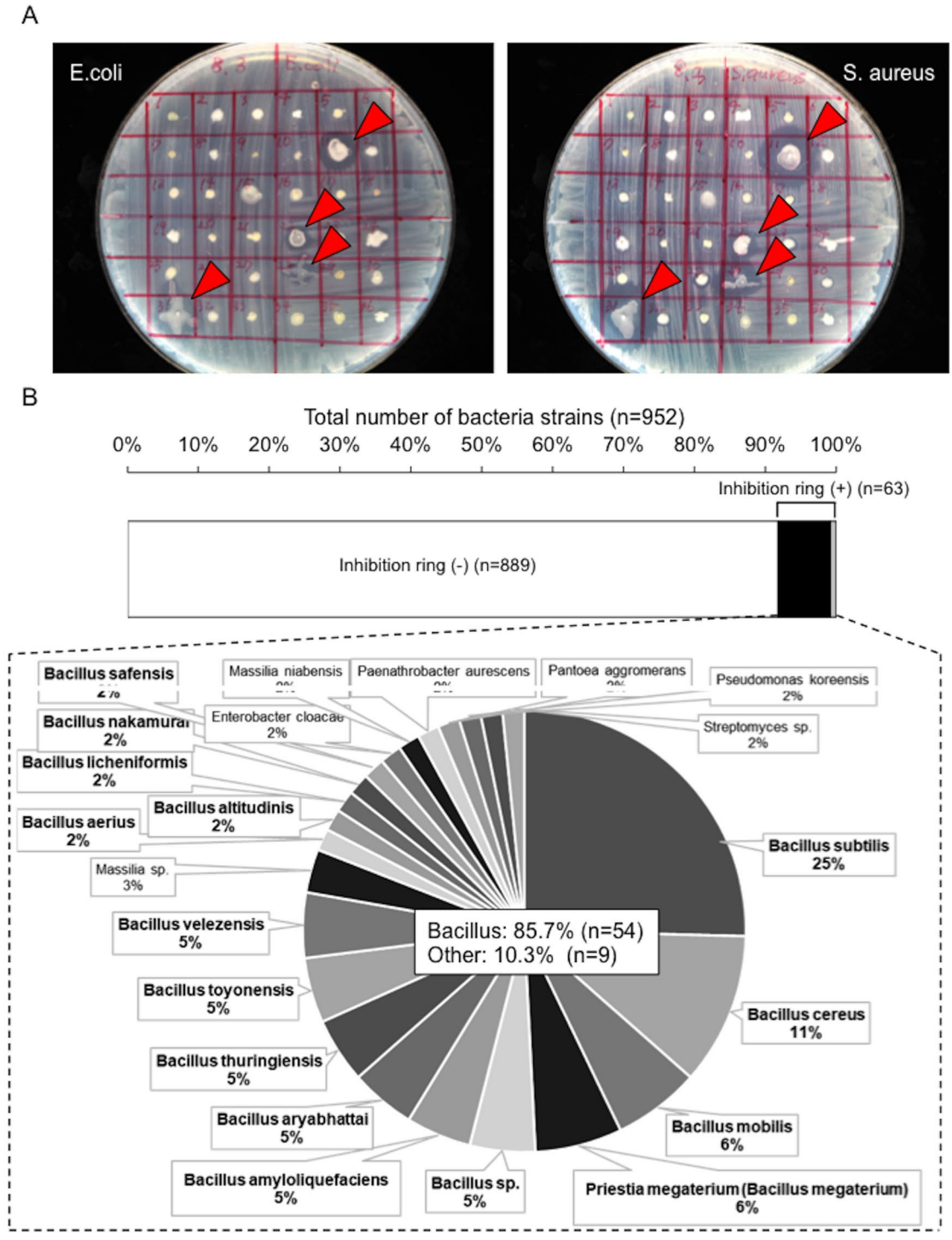
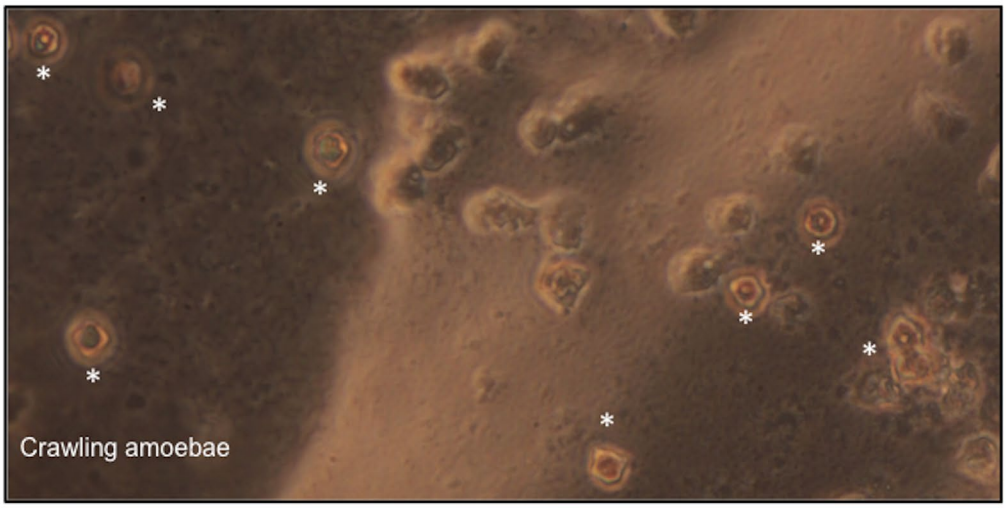

A
E.coli
S. aureus
B
Total number of bacteria strains (n=952)
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Inhibition ring (+) (n=63)
Inhibition ring (-) (n=889)
Bacillus safensis
Massilia niabensis
Paenathrobacter aurescens
Pantoea aggromerans
Pseudomonas koreensis 2%
Bacillus nakamurai 2%
Enterobacter cloacae 2%
Streptomyces sp. 2%
Bacillus licheniformis 2%
Bacillus altitudinis 2%
Bacillus aerius 2%
Bacillus subtilis 25%
Massilia sp. 3%
Bacillus velezensis 5%
Bacillus: 85.7% (n=54)
Other: 10.3% (n=9)
Bacillus toyonensis 5%
Bacillus cereus 11%
Bacillus thuringiensis 5%
Bacillus aryabhattai 5%
Bacillus mobilis 6%
Bacillus amyloliquefaciens 5%
Bacillus sp. 5%
Priestia megaterium (Bacillus megaterium) 6%

Figure 5

A



B