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Impact of dust on airborne *Staphylococcus aureus'* viability, culturability, inflammogenicity, and biofilm forming capacity

John Kerr White [a,b], Jeppe Lund Nielsen [b], Cecilie Møller Larsen [a], Anne Mette Madsen [a,*]

[a] The National Research Centre for the Working Environment, Lersø Parkallé 105, 2100, Copenhagen Ø, Denmark
[b] Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers Vej 7H, 9220, Aalborg Ø, Denmark

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen associated with soft-tissue infections commonly encountered in indoor and farm environments as a component of airborne dust, which can potentially deposit in the respiratory tracts of workers and residents. However, knowledge regarding the survival and inflammatory potential of *S. aureus* in airborne dust has not been described. The objective of this study was to obtain knowledge on whether the presence of dust during aerosolisation affects the culturability (ability to grow on agar plates), their biofilm forming capacity, viability (using a viability qPCR), and inflammatory potential (using a human granulocyte based assay), and whether time from aerosolisation to subsequent analyses (the resting time) affects these. Aerosols containing *S. aureus* (DSM6148) in the presence of sterilised airborne dust from a pig farm were found to have higher culturability, viability, inflammatory potential, and ability to form biofilm compared with *S. aureus* aerosols generated without airborne dust. When aerosols of *S. aureus* were generated without dust, they showed a reduction in the culturability, as well as the biofilm forming capacity and an extended resting time was associated with a reduction in culturability, and biofilm forming capacity. In contrast, no differences in the viability were observed in samples with different resting times. The lack of a significant effect of different resting times on viability, and the significant effect on culturability, suggests that the stresses of aerosolisation may induce a viable, but not culturable (VBNC) state in *S. aureus*. A synergistic effect was found between *S. aureus* and dust concerning their ability to induce inflammation. In conclusion presence of airborne dust during aerosolisation of *S. aureus* affects the culturability, biofilm forming capacity, and inflammatory potential, but not the viability of *S. aureus*. This is of importance in relation to hygiene as well as how exposure to *S. aureus* is measured.

1. Introduction

Microorganisms, both dead and alive, constitute a major component of dust particles in indoor (Gutarowska et al., 2018) and farm environments (Rintala et al., 2012). Although the majority of these species are non-pathogenic (Ling and Hui, 2019; White et al., 2019), they contain, among others, livestock-associated and community acquired methicillin-resistant *Staphylococcus aureus* (LA and CA-MRSA), both of which are increasing in prevalence in Denmark (DANMAP, 2019) and pose a potential occupational health issue. The level of dust within farms positively correlates with the concentration of airborne MRSA (Madsen et al., 2019), and exposure to airborne LA-MRSA in pig farms is a known risk factor for the nasal colonisation of LA-MRSA (Angen et al., 2017). In addition, airborne dust can settle on high contact fomites, such as door handles, table surfaces, and on workers' hair or clothing. Once on these fomites, *S. aureus* can be further spread through the environment via hand contact. Although there are rules for showering post occupancy in LA-MRSA positive farms (Sundhedsstyrelsen, 2016), transport of LA-MRSA from the farm environment to, for example, family relatives (Dorado-García et al., 2013), neighbours (Anker et al., 2018), and farmer homes (Van Cleef et al., 2015) has been reported. This might partly be related to the survival of LA-MRSA for several weeks within farm dust particles (Feld et al., 2018). In addition, several recent studies have shown that there is an increasing frequency of detecting airborne-MRSA from different indoor environments, such as hospitals and homes (Gandara et al., 2006; Gupta et al., 2015; Otter and French, 2010).

In the risk assessment of exposure to airborne infectious microorganisms such as *S. aureus,* it is important to consider whether the bacteria are viable, typically measured as the ability to produce colony

* Corresponding author.
E-mail addresses: xjkw@nrcwe.dk (J.K. White), jln@bio.aau.dk (J.L. Nielsen), amm@nrcwe.dk (A.M. Madsen).

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forming units (CFUs). However, if the bacteria are viable, but non-culturable (VBNC), or do not/cannot grow on selective media, then there is a risk of a false negative observations. Alternatively, with molecular tests, such as polymerase chain reaction (PCR), a signal coming from dead bacteria or exposed DNA may lead to false positives. To overcome these biases, it is possible to modify free DNA and DNA from cells with compromised cell walls by intercalating with DNA binding agents, such as propidium monoazide (PMA) or ethidium monoazide (EMA) (Emerson et al., 2017). This reaction is catalysed by exposure to light and prevents the exposed DNA from being amplified during PCR, thus only allowing for the amplification of DNA present in intact cells. A comparison of samples with and without this treatment allows researchers to differentiate the relative proportion of damaged or dead cells to intact cells.

In addition to the importance regarding the detection of airborne pathogens in indoor and occupational environments, knowledge on the inflammatory potential of aerosols containing *S. aureus* and the impact of dust is needed. Earlier research has shown that exposure to bioaerosols can be linked to worsened lung function in workers (Heldal et al., 2003) and higher exposure to microbes in aerosols has been found to correlate with an increased inflammatory potential (Timm et al., 2009). Previous work has identified staphylococcal enterotoxin genes in dust containing *S. aureus* (Ludwig et al., 2017), but the inflammatory potential of airborne *S. aureus* in the presence of dust thus far has not been studied.

S. aureus is also able to form biofilms, defined as a community of bacteria which have irreversibly attached themselves to a surface, abiotic or biotic, and secrete extracellular polymeric substances (EPS) resulting in higher antibiotic resistance and prevention of phagocytosis (de la Fuente-Núñez et al., 2013; Høiby et al., 2010; Limoli et al., 2015). *S. aureus* has multiple molecular triggers for biofilm production (Archer et al., 2011) and biofilms containing *S. aureus* have been found in the airways of patients with chronic lung disease (Cullen and McClean, 2015; Shimizu et al., 2015). In addition, it is well established that *S. aureus* is one of the many aetiological agents causing pneumonia in humans (Lowy, 1998). Previous studies on the survival of bacterial pathogens on surfaces, such as face masks, have shown evidence for a positive effect of the presence plant biomass dust for bacterial survival and biofilm formation (Majchrzycka et al., 2018, 2017), and the presence of road dust has a positive effect on the growth of bacteria in nutrient poor media (Suraju et al., 2015). Therefore, we are interested in the potential for the formation of a *S. aureus* biofilm after aerosolisation and its dependency to the presence of dust.

For *S. aureus* in pig farm dust, the die-off rate is initially faster during the first days after sampling of airborne dust and subsequently declines (Madsen et al., 2019). Whether this is due to different ages or sources of *S. aureus* isolates in the dust remains unknown. However, due to its ability to survive or avoid desiccation in farm dust (Feld et al., 2018; Madsen et al., 2019), we hypothesise that stable dust might act as a nucleation particle for the formation of biofilms in the pig farm environment, allowing *S. aureus* to persist until it is dispersed and potentially enters a host.

The aim of this study was to obtain knowledge on the impact of airborne dust in regards to the culturability of *S. aureus* (determined by colony forming unit (CFU) enumeration), viability (tested using a viability qPCR), inflammogenicity (using a granulocyte based assay), and biofilm forming capacity post-aerosolisation.

2. Materials and methods

In this study, aerosols containing *Staphylococcus aureus* were generated in the presence or absence of sterile pig farm dust. After the collection of the aerosols, the culturability, biofilm forming capacity, viability, and inflammatory potential were assessed using the methods stated below.

2.1. *Staphylococcus aureus* suspensions and bubble generation

Staphylococcus aureus spp. *aureus* Rosenbach 1884 (DSM 6148, DSMZ, Germany), an antibiotic sensitive strain, was grown overnight in Tryptic Soya Broth (TSB) (Oxoid, United Kingdom) at 37 °C under constant agitation. Cells were diluted to the target concentration of 7.5 $\times 10^5$ cells/mL in 230 mL of sterile isotonic solution 0.9% (wt/vol) NaCl after cell counting using a haemocytometer (Reichert, Germany).

Afterwards, the solution was loaded into a custom bubble generator designed as detailed elsewhere (Reponen et al., 1997). Bubble generation was achieved by passing sterile air into the bacterial suspension at a flow rate of 2 L/min. Post bursting of the bubbles, the resulting droplets (hereby referred to as aerosols) were dried and ejected from the custom bubble generator by the constant flow of sterile air above the liquid line at a flow rate of 10 L/min.

2.2. Collection and aerosolisation of dust

Sedimented dust particles from multiple pig stables were collected over a period of 14 days using sedimentation boxes. Culturing of the dust showed high concentrations of *Aerococcus viridans* and various *Staphylococcus* species. Prior to this study, collected dust particles were then sterilised using 50 kGy of gamma radiation (Sterigenics, Denmark). After sterilisation, the dust was noted to have a concentration of 123.34 endotoxin units (EU)/mg dust as measured using the limulus assay as described elsewhere (Frankel et al., 2012). Sterility of the dust was confirmed by plating, where no colony forming units (CFUs) were observed after serial dilution of 0.5 mg dust.

During experiments when bacterial aerosols were exposed to airborne dust, the sterilised stable dust was added to the aerosol chamber by using a Fraunhofer microdosing system (Fraunhofer, Germany). Sterile air was passed through the Fraunhofer microdosing system at an air flow rate of 2 L/min to inject dust. The dust was composed of particles of multiple aerodynamic diameters. During aerosolisation of dust and bacteria, the geometric mean diameter (D_g) of the particles within the chamber ranged between 0.291 and 0.320 μm. The D_g of particles within the chamber during aerosolisation of dust without the presence of bacteria ranged between 0.307 and 0.321 μm.

2.3. Aerosol sampling

A GRIMM dust monitor (GRIMM, Germany) was used to monitor the size-separated concentration of aerosols and dust within the chamber. Sampling began once the aerosol chamber was saturated with aerosols, meaning that the concentration of aerosols within the chamber did not increase as monitored by the GRIMM dust monitor. The D_g of particles within the aerosol chamber before sampling ranged between 0.290 and 0.321 μm. The D_g of particles within the aerosol chamber while aerosols of *S. aureus* were being generated ranged between 0.291 and 0.318 μm. Sampling occurred by using four (4) gesamtstaubprobenahme (GSP; BIG Inc., USA) samplers mounted with polycarbonate filters (poresize 1 μm; CIS by BGI, Inc., Waltham, MA, USA) for the collection of aerosols using a flow rate of 3.5 L/min for 30 min. In total, 40 samples were taken. For testing the inflammatory potential, aerosols were generated and sampled ($n = 16$) as described above, but for 2 h. These samples were also used to obtain knowledge on the culturability, viability, and biofilm forming capacity. In addition, dust was generated for 2 h without the presence of *S. aureus* using GSPs mounted with 37 mm 1 μm pore Teflon filters (Merck Millipore, Ireland) pre-weighed in a climate-controlled weigh-room ($n = 8$). Collected dust was allowed to acclimatise in the climate-controlled room and weighed the following day before being extracted (see description below).

2.4. Sample extraction

Once sampling was completed, the GSPs and aerosol and dust

generating air flows were turned off. When the concentration of aerosols in the chamber returned to baseline (approximately 30 min), the GSPs were removed from the chamber. Bacteria and dust on the filters from the GSPs were extracted in 5.0 mL of extraction fluid (0.85% NaCl and 0.05% Tween80, or 0.85% NaCl and 0.001% Tween80 for samples used for testing inflammatory potential) by shaking at 500 rpm for 10 min either immediately after removal from the aerosol chamber, which took approximately 30 min, (filters 1 and 2) or 3 h post sampling (filters 3 and 4). The time from the end of sampling to extraction is henceforth referred to as the resting time, and the effect of resting time, 30 min versus 180 min, was studied. During the study, the median temperature in the aerosol chamber ranged from 21.5 °C to 24.8 °C, and the median relative humidity (RH) ranged from 36.4% to 77.7%. No significant effect of relative humidity and temperature was observed on culturability, viability, biofilm forming capacity, inflammogenicity, and therefore data regarding this is not shown. A schematic overview of the aerosol generation, sampling, and subsequent analyses is shown in Fig. 1.

2.5. Culturability and identification

Homogenised bacterial suspensions were plated onto nutrient agar (NA) (Oxoid, United Kingdom) plates in duplicate and were incubated at 37 °C for 18–24 h before enumeration. Colony forming units (CFUs) were confirmed to be *S. aureus* by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany) using the B-dal standard library as described elsewhere (Feld et al., 2018).

2.6. Biofilm forming ability

Biofilms were grown and analysed using a crystal violet (CV) assay (O'Toole, 2011). In brief, 1.0 mL of the extracted bacterial suspension was added to 9.0 mL of TSB, vortexed, and then aliquots of 100 μL of this

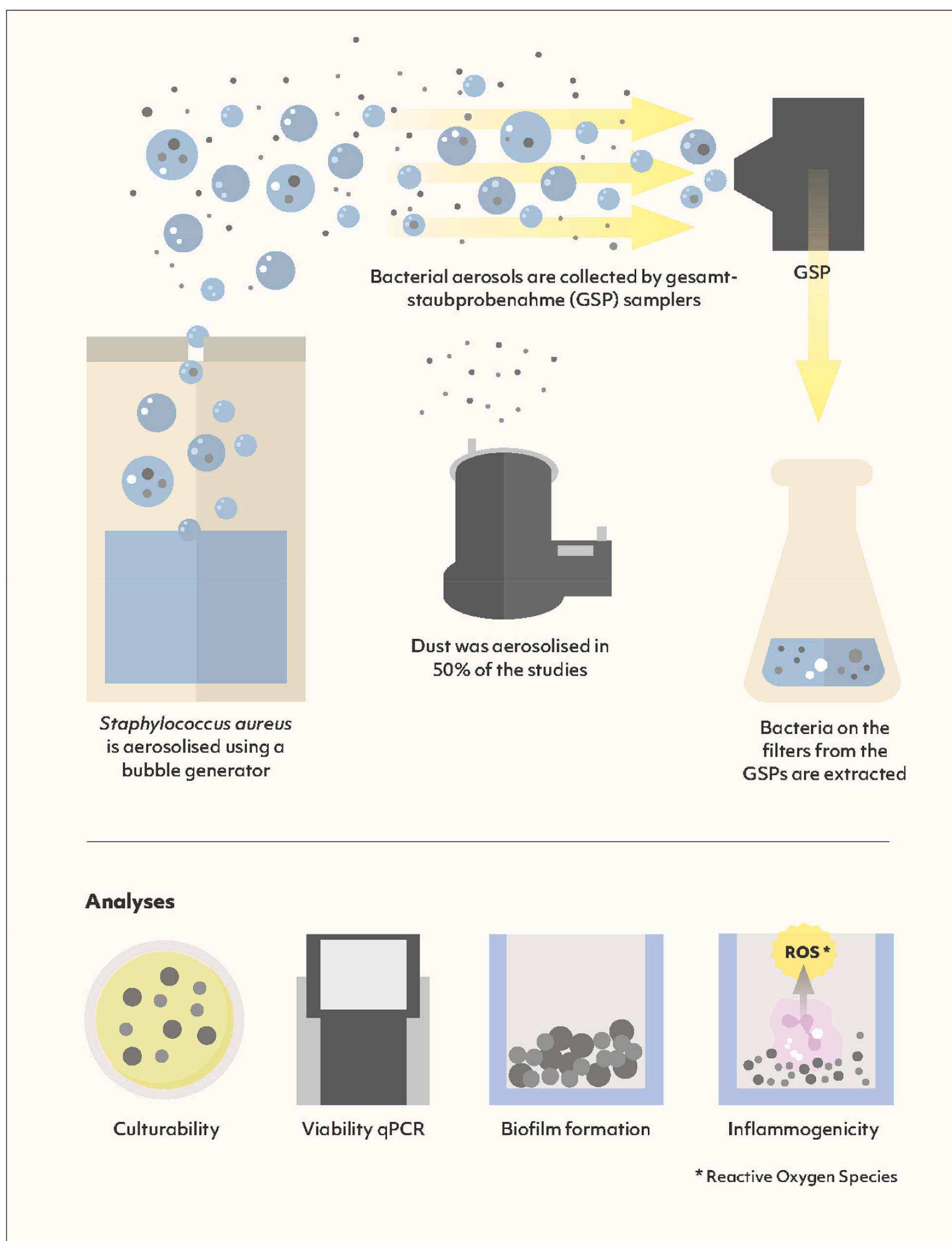

Fig. 1. Overview of sample generation and subsequent analyses performed on collected aerosols. ROS: Reactive Oxygen Species.

suspension were dispensed to a 96-well flat bottom clear microtiter plate (Corning, USA) and incubated at 37 °C for 24 h. Positive growth controls included 1.0 mL taken from the bubble generator, and an overnight culture of *S. aureus.* For each sample, 1.0 mL of each control was added to 9.0 mL of TSB. A negative control, consisting of un-inoculated TSB was included in every run. After incubation, supernatant and planktonic cells were removed and each well was washed with 125 μL of sterile water. Biofilms were stained by using 125 μL of 0.1% CV (Sigma Aldrich, Germany) in water and stained for 15 min. After staining, the unbound CV was removed and excess CV was removed by washing the wells with sterile water until the wash was clear before being left to dry. Bound CV was eluted the next day using 30% (v/v) acetic acid in water (Sigma Aldrich) and transferred to a new 96-well plate and the OD_{600} was measured using an Epoch microplate spectrophotometer (BioTek, USA), using the aforementioned negative controls as blanks. An average of the OD_{600} (n = 8) was used for quantifying the amount of biofilm formed in each sample during statistical analysis.

2.7. Viability qPCR

Investigation of bacterial cell wall integrity was done using a commercial viability qPCR (vPCR) kit (Biotium, USA). From the same GSP sample, two aliquots of 500 μL were taken. One bacterial suspension was treated with 5 μL of 2.5 mM PMAxx (Biotium) while incubating for 10 min in the dark under gentle agitation before being photolysed for 15 min using the PMA-lite photolysis system (Biotium), as described elsewhere as recommended by the manufacturer. Viability controls of heat-killed and viable *S. aureus* in log-phase growth with and without PMAxx treatment were included. DNA was then extracted from samples using the QIAamp DNA mini kit (Qiagen) following the manufacturer's protocol for isolation of genomic DNA from Gram-Positive bacteria using a lysis buffer (8×10^5 U/mL lysozyme; 20 mM Tris HCl; 2 mM EDTA; 1.2% TritonX).

vPCR was run according to the manufacturer's protocol with the recommendations for ROX based on the qPCR machine (Viia7, Thermo Fisher Scientific, USA) taken into account. For each run, a no template control was included. Viability analysis was done by comparing the difference in cycle threshold number (ΔC_t) for the same GSP sample treated with, or without PMAxx. The ΔC_t is an inverse relationship, meaning that the greater the ΔC_t value, the lower the viability within that sample.

2.8. Total inflammatory potential

Measurement of the total inflammatory potential (TIP) was performed using a chemiluminometric based assay using granulocyte-like cells differentiated from HL-60 cells. Upon exposure to inflammogens, the granulocytes produce reactive oxygen species (ROS) which are quantifiable by a luminol-dependent reaction. Firstly, the HL-60 cells were differentiated by adding all-*trans* retinoic acid (Sigma Aldrich, Germany) to the growth media RPMI-1640 (Biological industries, USA) and incubating the cells for 6–7 days without changing the media. The cells were seeded at 3×10^5 cells/mL and incubated at 37 °C with 5% CO_2 as described previously (Frankel et al., 2012). To test the inflammatory potential of the aerosolised bacteria and/or dust, 50 μL of granulocyte cell suspension and 100 μL of extracted bacteria were inoculated into each well in a white microtiter plate (Thermo Fisher Scientific) together with 30 μL of Hanks' balanced salt solution (HBSS), 10 μL of luminol, and 10 μL of human plasma. Each sample was tested in duplicate. The chemiluminescent reaction was measured at 37 °C in an ORION II microplate luminometer (Berthold Detection Systems, Germany) which measured relative light units (RLUs) for 1 s every 120 s over a 180-min period. To account for the reactivity level of the samples, a reference standard of 0.001% Tween80 in sterile water was included in every run. To calculate the TIP, the total number of RLUs per sample was summed and normalised by the reference. Subsequently, the TIP values were adjusted to the amount of dust collected for each sample based on the gravimetric data obtained from the Teflon filter samples.

2.9. Statistics

Statistical analyses regarding the two main exploratory variables, the presence of dust and the length of resting time (defined as the time from the end of sampling to extraction) on culturability, viability, biofilm formation, and inflammation were performed using R version 4.0.2 (Team, 2015) in RStudio (RSudio Team, 2016). A total of 56 samples were included for the statistical analysis of the culturability, viability, and biofilm forming capacity, 28 aerosolised with dust and 28 without dust. For the analysis the inflammatory potential, 24 samples were used, 8 were *S. aureus* aerosolised with dust, 8 were *S. aureus* without dust, and 8 were of dust aerosolised without bacteria.

For each studied parameter (culturability, biofilm forming capacity, viability, and inflammatory potential), we created linear mixed-effect models where the presence of dust and the resting time were fixed effects. To account for day-to-day variation, we included the sampling day as a random effect. In addition, the potential interaction between dust and resting time was also included in the models. Models were built and analysed using the R packages lme4 (Bates et al., 2015) and car (Fox and Weisberg, 2019). Residual Q-Q plots confirmed the diagnostic of the linear-mixed effect models. All data was log-transformed before analysis. All data visualisations were performed in R using the R package ggplot2 (Wickham, 2016).

3. Results

3.1. Culturability of S. aureus aerosols

S. aureus aerosolised without dust and extracted 3 h after sampling had the lowest concentration of culturable *S. aureus* in the air while *S. aureus* aerosolised with dust and extracted immediately after removal from the aerosol chamber (approximately 30 min after sampling) had the highest concentration of culturable *S. aureus* in the air (Fig. 2). The resting time, defined as the time taken after sampling of aerosolised *S. aureus* on filters, either with or without simultaneous aerosolisation of dust, until the extraction of bacteria from the filters, was observed to have a significant impact on the number of culturable *S. aureus* on nutrient agar (NA) plates, where the greatest number of CFUs formed in samples with a shorter resting time ($p < 0.001$). The analysis showed that the effect of the length of the resting time on the concentration of culturable *S. aureus* in air was not affected by the presence of aerosolised dust (i.e., no significant interaction term between presence of aerosolised dust and resting time variables; $p = 0.186$).

3.2. Viability of S. aureus

The difference in cycle number threshold (C_t) after qPCR between samples treated with PMAxx and a control without, hereby referred to as ΔC_t, was used for the analysis of viability post aerosolisation. The ΔC_t is used to analyse the relative difference in free or exposed *S. aureus* DNA present after sampling, and as the ΔC_t increases, fewer cells are considered viable.

Samples which were aerosolised simultaneously with dust generally had lower ΔC_t values compared to samples aerosolised without dust (Fig. 3). The presence of dust was noted to have a significant effect on the ΔC_t values ($p = 0.033$) whereas the resting time had no significant effect ($p = 0.483$). No significant interaction between presence of dust and resting time was observed regarding the viability of airborne *S. aureus* in the air ($p = 0.133$).

3.3. Biofilm formation

Both the presence of dust and the resting time were found to have a

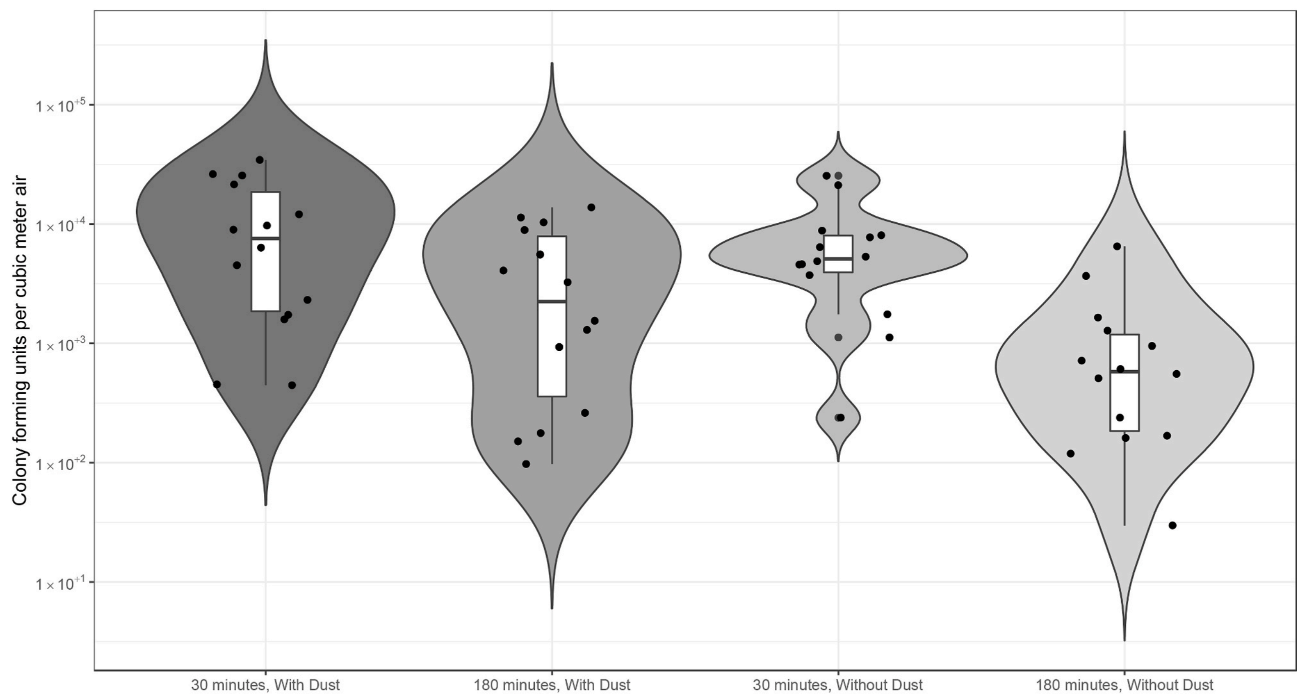

Fig. 2. Tailed-violin and boxplots showing the effect of resting time (30 min vs 180 min) and presence of dust on the number of culturable *S. aureus* colonies per m^3 air post aerosolisation. The boxplots bound the interquartile range (IQR) divided by the median, the whiskers extend to 1.5 × IQR beyond the box, while the violin plots show the probability density of the data.

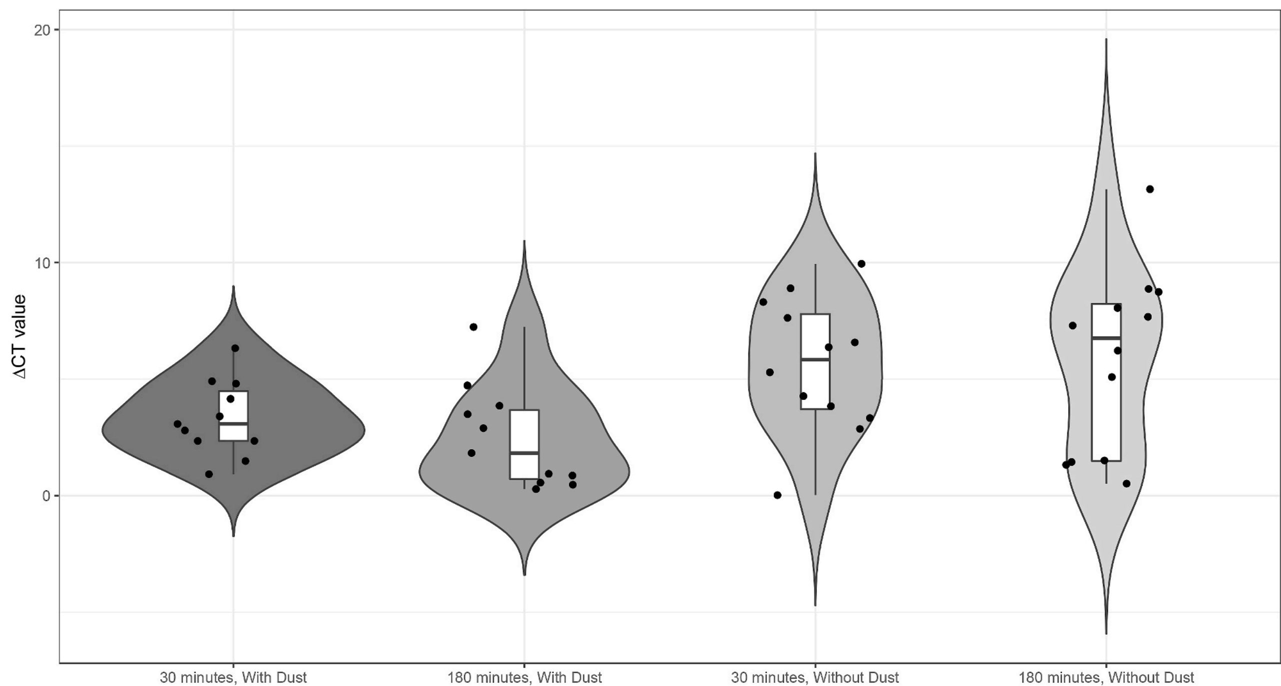

Fig. 3. Tailed-violin and boxplots showing the change in cycle threshold (ΔC_t) number after viability qPCR for *S. aureus* aerosols in regards to the effect of resting time (30 min vs 180 min) and presence of dust during aerosolisation. The boxplots bound the interquartile range (IQR) divided by the median, the whiskers extend to 1.5 × IQR beyond the box, while the violin plots show the probability density of the data. The ΔC_t is used to analyse the relative difference in free or exposed *S. aureus* DNA present after sampling, and as the ΔC_t increases, fewer cells are considered viable.

significant effect on the amount of biofilm formed by *S. aureus* after aerosolisation ($p < 0.001$ & $p < 0.001$, respectively), with most biofilm formed after aerosolisation with dust, and with a short resting time (Fig. 4). The analysis showed that the effect of the length of the resting time on the amount of biofilm formed by *S. aureus* was nearly affected by the presence of aerosolised dust (i.e., no significant interaction term between presence of aerosolised dust and resting time variables; $p = 0.093$).

The morphology of the biofilms which formed after aerosolisation were markedly different in shape and form compared to the controls. In

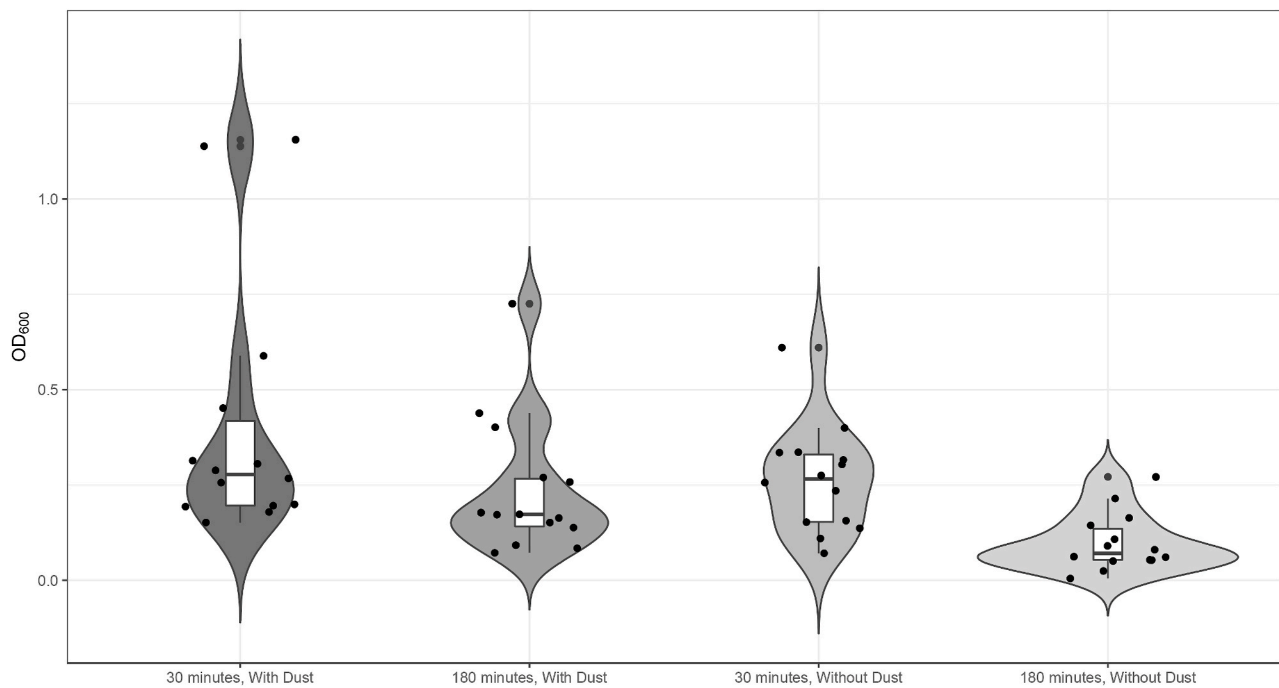

Fig. 4. Tailed-violin and boxplots regarding the biofilm forming capacity of *Staphylococcus aureus* as measured by the average OD_{600} absorbance of biofilms produced in regards to the effect of resting time (30 min vs 180 min) and presence of dust during aerosolisaion. The boxplots bound the interquartile range (IQR) divided by the median, the whiskers extend to 1.5 × IQR beyond the box, while the violin plots show the probability density of the data.

these samples the biofilms were morphologically more structured with stalked, bulbous extracellular polymeric substance (EPS)-encapsulated cells compared to the biofilms formed from an originally planktonic culture (Supplementary File S1).

3.4. *Inflammatory potential of aerosolised bacteria*

S. aureus which had been aerosolised without dust, showed almost no inflammation in the granulocyte-assay. In contrast, the simultaneous aerosolisation of *S. aureus* with dust showed a significant increase in inflammogenicity ($p = 0.003$) compared with *S. aureus* or dust aerosolised individually (Fig. 5). No difference in the total inflammatory potential (TIP) of *S. aureus* was observed between samples with short (30 min) and long (180 min) resting times ($p = 0.670$).

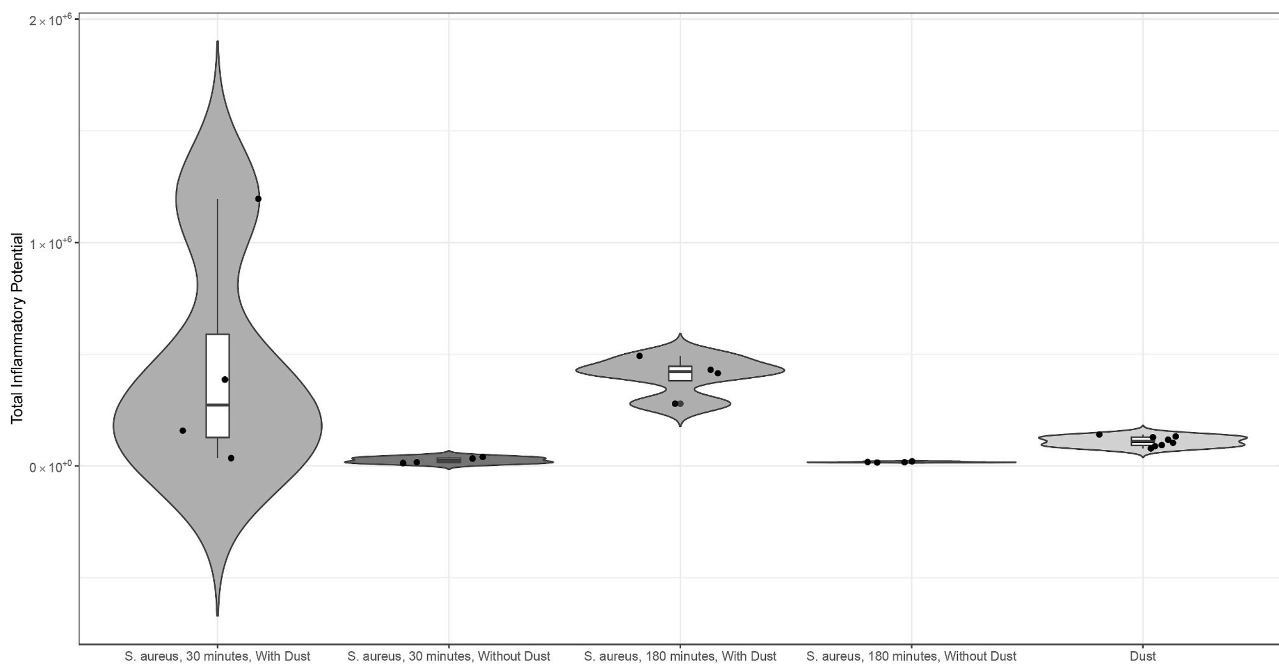

Fig. 5. Tailed violin and boxplots showing the total inflammatory potential (TIP) of the aerosols collected over a period of 180 min containing pure *Staphylococcus aureus* (n = 8), *S. aureus* with dust (n = 8), and dust (n = 8). The boxplots bound the interquartile range (IQR) divided by the median, the whiskers extend to 1.5 × IQR beyond the box, while the violin plots show the probability density of the data.

4. Discussion

Aerosols containing mucus or exfoliated skin particles (Zhao et al., 2014), have been suspected to potentially be a transmission vector for the spread of *S. aureus* in the medical (Dansby et al., 2008; Schultsz et al., 2003), home (Madsen et al., 2018), and farm (Angen et al., 2017) environments. Aerosols containing *S. aureus* therefore represent an important ecological reservoir of cells, which require further studying whether aerosolisation itself has an effect on the survival and/or pathogenicity of the bacterium.

Therefore, we have investigated the effect of exposing *in vitro* generated aerosols containing a monoculture of *S. aureus* to airborne dust particles from a pig farm stable. The culturability, viability, inflammogenicity, and biofilm forming capacity of aerosolised *S. aureus* were investigated under standardised laboratory conditions where physiological and osmotic stresses were controlled. In addition, the bacteria being aerosolised were in similar stages of growth and were grown from the same strain, which removes some uncertainty regarding potential confounding factors, such as differences between strains (Cucarella et al., 2001).

According to the results of this study, simultaneous aerosolisation of *S. aureus* with dust, and time from sampling on filters to extraction (resting time) had a significant impact on biofilm formation. In addition, these two factors affected the culturability of *S. aureus.* The greatest number of CFUs formed if the samples were aerosolised simultaneously with dust and extracted from the filter immediately post removal from the aerosol chamber and the fewest number of CFUs formed from samples aerosolised without dust and extracted 180 min post aerosolisation. While previous research has shown that *S. aureus* can be cultured from dust (Feld et al., 2018; Lidwell and Lowbury, 1950; Wilkoff et al., 1969), this study shows that *S. aureus* has a nearly significant higher culturability when it is in dusty environments, in comparison to non-dusty environments (Fig. 2).

For some bacteria, aerosolisation is a major stressor, as during a study on the survival of gram-negative bacteria, they are subject to drying whilst airborne, which in turn reduces their survivability (Potts, 1994). While the gram-positive *S. aureus* is known to have a relatively high desiccation tolerance, a decrease in the survival of laboratory cultured *S. aureus* after drying for several days in microtiter plates has been observed (Chaibenjawong and Foster, 2011). In this study, all the samples had positive ΔC_t values, indicating a reduction in the number of viable *S. aureus.* However, we also found that samples aerosolised with dust, had a significantly lower ΔC_t value than samples aerosolised without dust, which indicates that a larger proportion of *S. aureus* aerosolised with dust were viable compared with *S. aureus* aerosolised without dust. Additionally, according to the vPCR results, there was no significant difference in the viability between samples with resting times of 30 min or 180 min. The vPCR results, coupled with decrease in culturability, suggest that the stresses of aerosolisation might induce a VBNC state in *S. aureus*. Similar results have previously been found after the aerosolisation of gram-negative bacteria, where culturability of the aerosolised bacteria dropped rapidly while the viability (as measured using staining and microscopy) remained consistent (Heidelberg et al., 1997). This potentially has broad implications as cells which are in state of dormancy can withstand certain environmental stresses, such as presence of antibiotics, which would normally kill metabolically active cells (Lewis, 2010). However, it should be noted that vPCR is based on the assumption that non-viable cells will have damaged or fragmented cell walls (Emerson et al., 2017). For cells which were dead, but had an intact cell wall, analyses by vPCR might give results indicating that the cell population was viable. In addition, cells which were viable, but with damaged cells walls would have been assumed to be non-viable.

VBNC cells, which can be considered as persister cells, form an important ecological reservoir responsible for persistent and recurrent infections in humans (Hu and Coates, 2012; Li et al., 2014). The presence of *S. aureus* persisters is not only a concern in the clinical environment; in Denmark, the prevalence of MRSA and methicillin-susceptible *S. aureus* (MSSA) in the pig farm environment has been increasing, with 88% of randomly selected herds testing positive for MRSA in 2016, an increase from 68% of selected herds in 2014 (DANMAP, 2017). In addition, MRSA-negative human volunteers who entered and performed various work tasks for 1 h in a MRSA-positive pig farm were found to be MRSA-positive upon leaving, indicating that MRSA and MSSA are airborne in the pig farm environment (Angen et al., 2017).

In occupational and indoor exposure studies, airborne *S. aureus* detected using PCR-based methods (Van Cleef et al., 2016) allows for the detection of low-abundant cells, but gives limited knowledge on the viable microbial burden faced, whereas culture-based methods give insights on the quantity of the microbial burden (Masclaux et al., 2013), but are unable to detect the fraction which remain VBNC. The results from this study suggest that the "true" exposure to viable *S. aureus* aerosols might be much higher than previously thought, as no significant difference in viability between samples was observed (Fig. 3), even though the culturability decreased over time (Fig. 2).

Similar to the culturing results, the simultaneous aerosolisation of dust and *S. aureus* and a short resting time post sampling caused the greatest biofilm formation while aerosolisation without dust combined with long resting time post sampling caused the least biofilm formation. The impact of resting time is of importance as dust containing *S. aureus* may settle on surfaces, such as high contact fomites. Furthermore, the morphology of the biofilm was visually different from the biofilm produced by an *S. aureus* culture originally grown planktonically in the absence of dust with the aerosolised samples, with or without dust, having a non-homogenous, uneven surface morphology. It is possible that the difference in biofilm morphology could be due to changes in gene expression of the bacteria after they were aerosolised. In a different study looking at the changes in gene expression in *Escherichia coli*, it was found that multiple genes, including DNA damage and cold-shock associated proteins, were up and down regulated during aerosolisation (Ng et al., 2018). Therefore, it is likely that the molecular reason behind this difference in biofilm formation was due to a changed metabolism induced during or shortly after aerosolisation.

During this study, we exposed human granulocytes to aerosolised *S. aureus* with and without dust, and dust alone. When *S. aureus* was aerosolised without dust, almost no inflammation occurred in the cell assay. Although *S. aureus* is well known to induce an inflammatory response, the reason for the lack of an immune response in this assay could have been due to the relatively low concentration of *S. aureus* cells per well of the assay plate and the production of superoxide dismutases (SODs), which degrade ROS (Guerra et al., 2017). The production of SODs by *S. aureus* is a strategy to avoid degradation by the host immune system. The farm dust used in this study was noted to be inflammogenic, due to the presence of endotoxin, a well-known component of farm dust. Interestingly, the simultaneous aerosolisation of *S. aureus* and dust showed a 20-fold increase in inflammatory potential compared with *S. aureus* aerosolised alone, and a four-fold increase in inflammatory potential compared with aerosolisation of dust without bacteria. These results suggest the presence of a synergistic effect of the simultaneous aerosolisation of *S. aureus* and dust. Bacterial pathogens, such as *S. aureus* are known to cause inflammation in hosts (Liu et al., 2018; Lowy, 1998), and exposure to airborne microbial inflammogens or dust particles, has been associated with worsened lung function (Karottki et al., 2014).

The results from this study have shown concordance in regards to the culturability and biofilm forming capacity of *S. aureus*. We observed a higher culturability and more biofilm forming when aerosols were generated in the presence of dust and had a short resting time. A lowered culturability was observed when aerosols were generated without dust and had a long resting time. However, the molecular methods used in this study suggest that while the simultaneous aerosolisation of dust increases the inflammatory potential and viability, there is no difference

in the viability or inflammatory potential of the *S. aureus* aerosols with differing resting times. Traditionally, culture-based method have been used for conducting research on exposure to bacterial and fungal species, but these do not take into consideration the presence of VBNC microorganisms, which are still potentially able to cause infection. Relying solely on culture-based methods therefore are likely to underestimate the true level of exposure.

In many *in vitro* laboratory studies analysing the survival of aerosolised bacteria, aerosols are generated without dust or other particulate matter present (Alsved et al., 2018; Gut et al., 2016; Heidelberg et al., 1997; Perrott et al., 2017). This presents an issue as aerosols *in natura* are complex, composed of bacteria, viruses, organic dust, endotoxins, and other particulates, all of which can affect the culturability, viability, or inflammatory potential of the aerosols (Madsen et al., 2016; Pedersen et al., 2000; Uhrbrand et al., 2017). Therefore, this study reveals that it is important to consider the effect of stable dust on *in vitro* generated monoculture aerosols models.

While *S. aureus* is known to have high adhesion to various surfaces, including fomites (Simões et al., 2011) and medical equipment (Zheng et al., 2018), the exact reason as to why farm dust was observed to have significant effects on culturability, viability, and biofilm formation of aerosolised *S. aureus* remains unknown. Farm dust is complex, composed of microorganisms, endotoxin, faecal matter, feed materials, and animal dander (Donham et al., 1986). It is possible that the presence of skin particles and mucus within the dust had an effect, as *S. aureus* is a known commensal of the human and pig skin and nasal mucosa (Armand-Lefevre et al., 2005; Cuny et al., 2009; Frank et al., 2010; Noble et al., 1967). The recognition, and the subsequent reaction, to proteins or other biomarkers from some of these complex compounds (Foster et al., 2014; Pan et al., 2014; Patti et al., 1994) might have influenced the results we observed in this study. However, one of the major limitations of this study is that the effect of stable dust was only investigated for a single bacterial species. Environmental bioaerosols are often polymicrobial, composed of bacteria, fungi, viruses and other complex organic matter (Madsen et al., 2015; Uhrbrand et al., 2017). Another major limitation is that only pig farm dust was used in this study. Dust from other environments, such as from homes and hospitals, will not have the same chemical or microbiological fingerprint. It will be interesting to perform the same study with dust from other environments, such as hospitals and homes, where *S. aureus* is a health concern. While this study does provide evidence to the potential of *S. aureus* to enter the VBNC state and shows that dust is important for biofilm forming potential, more research on polymicrobial aerosols and their interaction with different dust types is needed to assess the true risk residents and workers may face when in areas of high concentrations of airborne microorganisms.

5. Conclusions

Exposure to airborne dust and the time from sampling to extraction from filter material were observed to have significant effects on the culturability and biofilm forming capacity of *S. aureus*, post aerosolisation. Aerosols containing both dust and *S. aureus* were observed to have a significantly higher inflammatory potential than when either component was aerosolised alone, suggesting a synergistic effect between the dust and *S. aureus*. In addition, this study suggests that the stresses of aerosolisation may induce the entering of a VBNC state, as there was a significant difference in the culturability, but not in the viability of samples extracted after 30 vs. 180 min. This study highlights that good hygiene practices with reducing the amount of dust may reduce the number of culturable *S. aureus*.

The results from this study underline and support the idea that good hygiene and reducing dust generation are important for the reduction of viable, inflammogenic, and potentially pathogenic staphylococci. In addition, when designing bioaerosol survival studies, the presence of other particulate matter i.e. dust, can have a significant effect on the culturability and inflammogenicity of bacterial CFUs and should be taken into account in the experimental design, particularly when considering using molecular or culture-based methods.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2020.113608.

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