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A cross sectional study on airborne inhalable microorganisms, endotoxin, and particles in pigeon coops – Risk assessment of exposure

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ABSTRACT

Pigeon breeding is associated with symptoms of the airways. The aim of this study is to illuminate the bacteriological and toxicological characteristics of airborne dust in pigeon coops. Airborne dust was sampled in 31 urban pigeon coops with homing and fancy pigeons, and following the dust was characterized. In total 141 different bacterial species were identified using MALDI-TOF MS, and of these 11 species are classified in risk group 2. Of the cultivable bacteria, *Staphylococcus equorum* was present in the highest concentration. Microorganisms in the dust were able to form biofilm, and the amount correlated positively with the number of bacteria. Next generation sequencing showed 180 genera with *Acinetobacter* in highest reads. On average 999 ± 225 ZOTUs were observed per sample with a Shannon-Wiener biodiversity index of 6.17 ± 0.24. Of the identified species the following have previously been suggested as causative agents of extrinsic allergic alveolitis: *Alcaligenes faecalis, Bacillus subtilis*, *Pantoea agglomerans*, *Sphingobacterium spiritivorum*, *Thermoactinomyces* sp., and *Streptomyces albus*. *Staphylococcus* was present on particles with sizes between 1.1 and > 7.0 μm with a geometric mean diameter of particles on 4.7 ± 1.1 μm. Concentrations of airborne endotoxin and dust were elevated compared to references, and the geometric mean concentrations were 102 EU/m^3 and 1.07 mg dust/m^3, respectively. Upon exposure to the airborne dust human granulocytes produced Reactive Oxidative Species during the first 5 min, and then no further reaction was observed. The concentrations of bacteria in general, *Staphylococcus* spp., and endotoxin and biodiversity were associated significantly with season, temperature and/or relative humidity, but not with type or density of pigeons. The bacterial composition and biodiversity indices were not affected by type of pigeon. In conclusion, the exposure to bacteria and endotoxin in pigeon houses should not be neglected in the evaluation of causative agents of airways symptoms among pigeon breeders.

1. Introduction

Several studies have shown that pigeon breeding is associated with symptoms of the airways (Calvert et al., 1999) diagnosed as extrinsic allergic alveolitis (EAA) which is also called hypersensitivity pneumonitis (Bourke et al., 1989; McSharry et al., 1985) and pigeon breeder's disease (Hargreave et al., 1966). Pigeon breeding is associated with the development of bronchiolitis (Perez-Padilla et al., 1996; Scadding, 1970), allergic asthma (Pelikan et al., 1983), lung inflammation, and nasal symptoms as obstruction and hyper-secretion (Pelikan and Pelikan-Filipek, 1983). Several causative agents have been described including feather mites (Colloff et al., 1997), particles and keratin from feathers (Tauer-Reich et al., 1994), pigeon droppings (Calvanico, 1986), and pigeon proteins (Bourke et al., 1989). Pigeon breeders with or without symptoms of the airways have antibodies to pigeon gamma globulin (Bourke et al., 1989). Animal breeding in general is associated with elevated exposure to microorganisms and endotoxin from bacteria, but even though pigeons and humans have lived in close proximity for thousands of years, bioaerosol exposure associated with pigeon breeding has not been well characterized. Exposure to airborne microorganisms has in several studies been ascribed as a causative agent of EAA (Ismail et al., 2006), and elevated endotoxin exposure with nasal symptoms

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(Douwes et al., 2000). Amongst workers in poultry farms, a high prevalence of toxic pneumonitis, airway inflammation, and chronic bronchitis has been found, and the suspected cause is a high exposure to endotoxin (Donham et al., 2000; Rylander et al., 2006).

Inflammation plays an important role in e.g. EAA, bronchiolitis, asthma and cardiovascular diseases. Production of reactive oxygen species (ROS) by exposed cells is considered a definitive marker of inflammation (Qu et al., 2017). The potential of an exposure to induce inflammation can be measured in vitro using an assay based on the production of ROS from granulocyte-like cells (Frankel et al., 2014; Timm et al., 2009) or interleukin release in a whole blood assay (Liebers et al., 2012).

The knowledge about bacterial species in pigeon coops is very limited, but coagulase-positive staphylococci, including *Staphylococcus aureus*, have been found in pigeon nares and/or cloacae (Kizerwetter-Swida et al., 2015). Microorganisms can be classified into risks groups where risk group 2 microorganisms can cause human disease, but it is unlikely to spread in the community as there is usually effective prophylaxis or treatment available (89/391/EEC, 2000). *Staphylococcus aureus* is a risk Group 2 human pathogen which in e.g. pig farms (Madsen et al., 2018) and cow barns (Locatelli et al., 2017) can be present in an antibiotic-resistant variant called MRSA.

The aerodynamic diameter of an inhaled particle determines where within the respiratory tract the particle will deposit and interact with host tissues. For development of EAA, particles have to be of a size which can enter the alveoli (McSharry et al., 2002).

Biofilm can be formed by bacteria in the respiratory tract (Percival et al., 2012), and has been observed in the airways of patients with e.g. chronic obstructive pulmonary disease (COPD) (Legnani, 2009) and chronic rhinosinusitis (Foreman et al., 2011). The ability of microorganisms to form biofilm is a virulence factor as it enables them to form persistent infections and contributes to protecting the bacteria from contact with antimicrobials and prevent the immune system from clearing the infection. In wounds biofilm formation may inhibit the effect of antibiotics and physical presence may obstruct wound healing (Vestby et al., 2020).

The aim of this study is to get knowledge on the potential exposure to airborne bacterial species, endotoxin, and airborne particles in pigeon coops, and the potential deposition of bacteria and particles in the human airways, and to obtain knowledge about the inflammatory potential and biofilm-forming capacity of airborne dust from pigeon coops. For the study, we have sampled airborne dust with microorganisms inside 20 pigeon coops with homing pigeons and inside 7 coops with fancy pigeons and outside 4 coops with fancy pigeons.

2. Material and methods

2.1. The 31 urban pigeon coops

Houses for pigeons are generally called lofts or coops. In this paper we use the term coop for the room in which pigeons are present mainly in the night. Some coops were inside another house, and some coops or houses were surrounded by an aviary. Most pigeon coops were open or semi open allowing fresh air to circulate. The coops were on average 14 m^2 and examples of pigeon coops and aviary can be seen in Fig. s1. The pigeon breeders (also called pigeon keepers or fanciers) entered the pigeon coops daily to inspect the pigeons, feed them, clean the coops, or let them out to fly. On average, the owners had 65.5 pigeons in total and 39.0 pigeons in the coops where the measurements were done (Table s1). The occupational hygienists visiting the coops filled out a questionnaire about the coops and the pigeons. The study was performed in 2016 in 10 pigeon coops (A-J) and in 2019 in 21 pigeon coops (1–21). The pigeons were domestic pigeons, including homing pigeons used for racing and fancy pigeons used for exhibition. The measurements were taken in pigeon coops in three regions of Denmark: Zealand, Funen, and Jutland in 15 towns.

Temperatures and relative humidity were measured every minute for 60 min using Tinytag Plus Data Loggers (Gemini data loggers, UK); this was done inside all coops and for coops 1–21 also outside the coops. This was done at the same time as sampling was done, and the average temperature and RH for each sampling session was calculated. The absolute humidity was calculated based on the measured relative humidity (RH) and the temperature (T) and a barometric pressure of 760 mm Hg, using this formula: Absolute Humidity (g/m^3) = (6.112 × e^[(17.67 × T)/(T+243.5)] × rh × 2.1674)/(273.15+T).

2.2. Sampling, extraction, and incubation of airborne bacteria

Air sampling was performed inside 27 pigeon coops and outside the chicken wire of four aviaries (Fig. s1). An overview of the samples taken can be seen in Table s2. As described in the following, sampling was performed using different methods.

Airborne dust was sampled using the Gesamtstaubprobenahme (GSP-sampler, CIS by BGI, INC Waltham, MA, USA) mounted with polycarbonate filters (pore size 1 μm, SUEZ – Water Technologies & Solutions, Feasterville-Trevose, PA, USA), and it sampled for 42–87min with a flow rate of 3.5 lpm. One to 5 h post sampling, the dust on the filters was extracted in 4.5 ml sterile solution (0.005% Tween 80, 0.85% NaCl) by orbital shaking for 15 min (500 rpm) at room temperature. Extracts were plated on Nutrient agar (in the following called NA; from Oxoid, Basingstoke, England) containing 50 mg/l actidione (cycloheximide, Serva, Germany) and incubated at 25 °C and for coop 1–21 also at 55 °C for 10 days. Bacteria counted on NA are called bacteria while bacteria on other media are named by an abbreviation of the used agar. For replating and biofilm formation, extracts were aliquoted in 1.0 ml volumes in cryotubes (Sigma Aldrich, Germany) with 0.5 ml of 85% glycerol. For next generation sequencing (NGS) and the total inflammatory potential (TIP) assay, extracts were aliquoted without glycerol. The samples were stored at −80 °C.

Samples were also taken using the Six-Stage Viable Andersen Cascade Impactor (ASCI; N6, Thermo Fisher Scientific Inc. Waltham, MA, USA; flow rate 28.3 lpm). The ASCI sampled bacteria present as or on particles of the following sizes: Stage 1: particles larger than 7.0 μm; Stage 2: between 4.7 and 7.0 μm; Stage 3: between 3.3 and 4.7 μm; Stage 4: 2.1 and 3.3 μm; Stage 5: 1.1 and 2.1 μm; Stage 6: 0.65 and 1.1 μm. The ASCI was mounted with six plates of *Staphylococcus* selective agar (SaSelect agar with peptone, salt mixture, antimicrobial and antifungal agents; SA; Bio-RAD, Marnes-la-Coquette, France) and in Coops 8–21 also with six plates of SSI Enteric agar which contains different salts, yeast extract, trisodium citrate, glucose, and L-tryptophan (SSI Diagnostica, Copenhagen, Denmark). For both media, sampling was performed for 8 min. In Coops A, C, D, and E sampling was also done on six Brilliance MRSA 2 plates (containing a peptone mix, carbohydrate, kaolin, salts and an antibiotic cocktail; in the following called MRSA-agar; Oxoid) for 13 min, and in Coops A to J on six NA for 4 min. The plates with SA, MRSA, and SSI-agar was incubated at 37 °C for 40–50 h and NA at 25 °C for 10 days.

In Pigeon Coops 1–21, sampling was also done using a MAS100 (sampling 100 lpm; Merck). The MAS100 sampler was mounted with NA plates and sampling was performed for 1 and 2 min, and one NA plate was incubated at 25 °C and the other at 37 °C for 10 days. The MAS100 was also mounted with MRSA-agar and in coops 8–21 also with SSI-agar plates. Samplings were done for 10 min, and the plates were incubated at 37 °C for 40–50 h.

Internal indoor office reference samples, taken during each of the four seasons using GSP samplers, were used as references. The samples were analyzed for bacteria on NA at 25 °C, endotoxin, biofilm forming capacity, and TIP.

Bacteria on all plates were inspected every day and isolates were counted and identified as described later.

2.3. Particle concentration

In pigeon coop A to J particle sizes and concentrations were measured using an optical particle sizer (Grimm; Grimm Aerosoltechnik, Model 1109, Ainring, Germany) with 31 channels between 0.25 and 32 μm in intervals of 6 s. The Grimm was placed 1 m above floor level. The concentration and the geometric mean aerodynamic diameter (D_{gae}) of airborne particles were calculated using the following formula: $D_{ae} = (D_1{}^{n1} \cdot D_2{}^{n2} \cdot \ldots \cdot D_6{}^{n6})^{1/N}$, where D_1 is the geometrical midpoint of the first interval, n1 is the measured number of bacteria in the interval, and N the total number of bacteria summed over each of the 6 intervals. In coop A measurement was done in the afternoon, night, and morning to obtain knowledge about diurnal variation in particle concentration. In addition, an indoor reference measurement was done in an empty office at the NRCWE three times in the autumn.

2.4. Identification of bacteria using MALDI-TOF MS

Bacterial isolates were identified using the MALDI-TOF MS Biotyper System (Bruker Daltonics, Bremen, Germany). In total 17,232 isolates were subjected for identification; for each NA and SA plate 10–200 isolates were randomly selected for identification; fewer bacteria grew on SSI-agar and all isolates were subjected for identification; for MRSA-agar only isolates appearing blue on the plates were selected for identification as MRSA bacteria appear blue on this medium.

For identification, a small amount of a bacterial colony was smeared onto the MALDI-TOF MS target plate (MSP 96 target polished steel BC; Bruker Daltonics) and 1 μL of 70% formic acid was added afterwards (called on-target extraction). The mixture was allowed to dry at room temperature before being overlaid with 1 μL of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid; Bruker Daltonics). Bacterial isolates that failed to give a positive identification were subsequently extracted using the ethanol extraction method as described in the supplementary file. A Microflex LT mass spectrometer (Bruker Daltonics) was used for the analysis and spectra were analyzed using Bruker Biotyper 3.1 software with the BDAL standard library. Extractions and MALDI-TOF analysis were repeated up to four times in attempt to obtain positive identifications of isolates. A bacterial test standard (Bruker Daltonics) was used to calibrate the instrument.

Isolates identified to be *S. aureus* were screened for their ability to grow on MRSA-agar.

2.5. Biofilm forming capacity

Particle suspensions from the GSP samples 1–21 with the glycerol were added to tubes with Tryptic soy broth (TSB; Oxoid, United Kingdom) in the ratio 1:10. After vortexing, aliquots of 100 μL from each suspension were dispensed into eight wells in a flat-bottom, clear microtiter plate (Corning, USA) and incubated at 37 °C for 24 h. After incubation, supernatant and planktonic cells were removed and each well was washed with 125 μL of sterile water. Biofilms were stained for 15 min using 125 μL of 0.1% crystal violet (CV; Sigma Aldrich, Germany) in sterile water (O'Toole, 2011). After staining, the unbound CV was removed by washing the wells with sterile water until the wash liquid was clear before being left to dry for 48 h. Bound CV was eluted using 30% acetic acid (Sigma Aldrich) and transferred to a new microtiter plate where the OD_{600} was measured using an Epoch microplate spectrophotometer (BioTek, USA). An average of the OD_{600} of the eight wells was used as an expression of the amount of biofilm formed. Values greater than that of the negative control (the TSB medium) were considered positive for biofilm formation.

2.6. Sampling of airborne dust for gravimetric and endotoxin analysis

Airborne dust was sampled using GSP-sampler, and the sampler was mounted with Teflon filters (Fluoropore, pore size 1 μm PTFE Membrane, Merck Millipore KGaA, Darmstadt, Germany), and it sampled for 60 min with a flow rate of 3.5 lpm. The mass of the dust collected on the Teflon filters was determined by weighing the filters before and after dust sampling. Before weighing, the filters used for collecting the dust were equilibrated at constant air temperature and humidity for 24 h. The limit of detection was 0.028 mg/m^3 dust. The detection limit was calculated as three times the standard deviation of 10 blanks and divided by the mean sampled volume.

2.7. Endotoxin extraction and analysis

The dust on the Teflon filters was extracted in 6.0 ml pyrogen-free water with 0.05% Tween 20 by orbital shaking (500 rpm) at room temperature for 60 min and centrifuging (1000×*g*) for 15 min. The supernatant was stored at −80 °C until they were analyzed (in duplicate) for endotoxin using the kinetic Limulus Amoebocyte Lysate test (Kinetic-QCL endotoxin kit, Lonza Pharma & Biotech, Walkersville, Maryland, USA). A standard curve obtained from an *Escherichia coli* O55:B5 reference endotoxin was used to determine the concentrations in terms of EU (11.0 EU ≈ 1.0 ng). The data are presented as EU/m^3 air, and the limit of detection was 0.05 EU/ml corresponding to a maximum detection limit of 1.42 EU/m^3. Two samples were also analyzed using the rFC assay (Lonza Inc.) as described by the supplier and elsewhere (Thorne et al., 2010).

2.8. Next generation sequencing

DNA from resuspended dust samples was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol with 400 μL of resuspended dust used for extraction. Extracted DNA was then amplified in a two-step PCR targeting the V4 variable region of the bacterial 16S rRNA gene. The primers targeting the V4 region were: 515F: GTGCCAGCMGCCGCGGTAA and 805R: GGACTACNVGGGTWTCTAAT (Caporaso et al., 2011). Amplicon library PCR was performed using 10 ng of extracted DNA as template per 25 μL PCR reaction (400 nM of each dNTP, 1.5 mM $MgSO_4$, 2mU Platinum Taq DNA polymerase High Fidelity, and 1x Platinum High Fidelity buffer (Thermo Fisher, USA)) and 400 nM of bar-coded library adapter pair (Illumina, USA). Thermocycler settings included initial denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 20s, 56 °C for 30 s, 72 °C for 60s and final elongation at 72 °C for 5 min.

All PCR reactions were run in duplicate and pooled. Amplicon libraries obtained were cleaned using AMPure XP bead protocol (Beckman Coulter, USA) with the following modifications: the sample/bead ratio was 5/4 and the purified DNA was eluted in 23 μL nuclease free water. Library concentrations were measured with Quant-iT HS DNA Assay (Thermo Fisher, USA) and quality checked using D1000 ScreenTapes (Agilent, USA). Samples were pooled in equimolar concentrations and the library pool was sequenced on the MiSeq platform (Illumina, USA) according to previous published procedure (Caporaso et al., 2012).

The 16S V4 amplicon data sequenced on the MiSeq platform was quality controlled and processed from raw data to Zero-Radius Operational Taxonomic Units (ZOTUs) at a 99% sequence similarity using AmpProc version 5.1.0.beta2.11.1. Taxonomy was assigned using SILVA (version 138) as the reference database for bacterial taxonomy (Quast et al., 2012). The obtained raw sequence data is available at the European Nucleotide Archive (ENA) under project accession number PRJEB45926 .

2.9. Inflammatory potential

Measurement of TIP was conducted using an assay based on granulocyte-like cells. The assay is based on the differentiated HL-60 cell line (Human Promyelocytic Leukemia cell line) which, upon exposure to inflammogens, will react by producing reactive oxygen species (ROS), quantifiable by a luminol-dependent chemiluminometric assay. The

cells were differentiated for 6–7 days by adding Tretinoin (ATRA) without changing the growth medium (RPMI 1640, Biological Industries, Cromwell, CT, USA). The cells were seeded at 3×10^5 cells/ml and incubated at 5% CO_2 at 37 °C as described previously (Frankel et al., 2014). All samples were diluted 20 times with sterile water with 0.85% NaCl. A volume of 100 μL of the diluted sample suspensions were in duplicate inoculated into 50 μL of the cells. The chemiluminesence reaction caused by sample activity was measured by a thermostated (37 °C) ORION II Microplate luminometer (Berthold Detection Systems, Germany), which measured relative light units per second (RLU/s) for 1 s every 120 s for 180 min. For every sample, accumulated RLU/s were calculated by summing the RLU/s measurements throughout the 180 min period. As positive references two endotoxin concentrations (1 and 5 EU/ml), zymosan (5 μg/ml), a suspension of *Aspergillus fumigatus* spores (10^6/ml) harvested from malt extract agar (Lu et al., 2020), and pig farm dust collected also for 1 h using GSP samplers (Madsen et al., 2019) were used. As negative reference the extraction solution diluted 20 times with sterile water were used. These references were used to see whether the cells reacted, and reacted consistently to the same level each time, and to test for contamination. To account for variations in sensitivities of the cells, all data were normalized to the reaction of the negative reference.

2.10. Treatment of data

All data are expressed as time-weighted averages/m^3 air. SAS 9.4 was used for statistical analysis of concentrations. The Pearson correlation coefficient between the log transformed exposure and climate factors was calculated. To see whether the climate or type of pigeon affected the log transformed concentrations of bacteria, endotoxin, and biofilm we analyzed this with Proc mixed model with random effect of place of sampling (inside the coop vs outside the aviaries). This was also analyzed for TIP but without any transformation of data. Indoor and outdoor climate were compared using paired *t*-test. Concentrations of bioaerosol components in the pigeon coops were compared with concentrations in the indoor reference samples using General Linear Models.

Analyses on the NGS data were performed in RStudio version 1.3.959, using R version 4.0.2. The following R CRAN packages were used: ampvis2 (Andersen et al., 2018), ggplot2 (Wickham, 2016), and vegan (Jari Oksanen et al., 2018). Microbial community composition and structure were explored using RDA plots on Hellinger transformed abundance counts. Statistical comparisons were run between clusters using the analysis of similarity (ANOSIM) function, part of the vegan package, according to Jaccard index values. For each cluster in the ANOSIM model a test statistic R value is given. A test statistic R value close to 1.0 suggest high dissimilarity between groups, while a test statistic R value close to 0.0 suggests no systematic differences between groups.

3. Results

3.1. The pigeon coops and climate

The average temperatures during each sampling period inside the pigeon coops were between 3.0 and 26.6 °C (average = 18), and the relative humidity between 32.1 and 97.0% (average = 64.4). Pairwise comparison between outdoor and indoor temperature ($p = 0.18$) and outdoor and indoor relative humidity ($p = 0.26$) showed no significant differences.

Eleven of the coops were placed in clubs with other coops while 20 were placed in private gardens close to the owners' home. All 20 coops with homing pigeons participated in national and international competitions, and 21 coops participated in national and international exhibitions (Table s1).

3.2. Airborne bacteria and endotoxin concentrations and factors affecting concentrations

All measured bioaerosol components in the pigeon coops were higher than in indoor office references (Table 1). The geometric mean (GM) concentration of bacteria as measured on NA using the GSP was 9000 cfu/m^3, and for the ASCI with SA the concentration was 2512 cfu of *Staphylococcus* spp./m^3 (Table 1). For four of these coops the measurements were done just outside the aviaries, and the concentrations of bacteria ($p = 0.047$; GM = 1652 vs 1.0×10^4 CFU/m^3) and *Staphylococcus* spp. ($p = 0.0002$, 340 vs 3504 CFU/m^3) were lower than inside the coops.

The 31 air samples were analyzed using the Limulus assay, and the GM concentration of airborne endotoxin was 102 EU/m^3 (Table 1), and it correlated with concentrations of bacteria, *Staphylococcus*, bacteria on SSI-agar (Fig. s2), numbers and mass of airborne particles, and with D_{gae} of airborne particles ($r = 0.92$; $p = 0.0002$, $n = 10$). For the four coops with measurements outside the aviaries the concentrations of endotoxin ($p < 0.0001$; GM = 4.7 vs 133 EU/m^3) were lower than inside the coops. Two samples from homing pigeon coops were also analyzed with the rFC assay. With the rFC assay the measured endotoxin concentrations were lower, and constituted only 40 and 57% of what was measured with the Limulus assay.

To see whether the climate or type of pigeon affected the concentrations of bacteria and endotoxin we analyzed this with Proc mixed model with random effect of place of sampling (inside the coop vs outside the aviaries). The concentration of airborne bacteria was

Table 1
Concentrations of bacteria, endotoxin, dust, and particles in pigeon coops, and aerodynamic diameter (D_{gae}), biofilm forming capacity, and total inflammatory potential (TIP) of airborne particles in pigeon coops.

Bioaerosols	Unit	Concentrations in pigeon coops		Office reference without people	
		GM 95% Confidence limits	Range n	GM 95% Confidence limits	Range n
Bacteria, NA, GSP	CFU/m^3	9000 [6516, 1.2 x10^4]	742 - 2.4x10^5 31	26 [22, 48]	5 - 54 4
Bacteria, NA, ASCI	CFU/m^3	3798 [2490, 5793]	732 - 5.5 7	nm	nm
Bacteria, SA, ASCI	CFU/m^3	2512 [1552, 4064]	181 - 1.6 x10^4 27	nm	nm
Bacteria, SSI, ASCI	CFU/m^3	18 [10, 35]	2-208 14	nm	nm
Endotoxin, GSP	EU/m^3	102 [70, 151]	2.1 – 745 28	0.25 [0.20, 0.32]	0.18-0.32 4
Dust, GSP	mg/m^3	1.07 [0.51, 1.64]	0.18 – 2.06 10	Bd	Bd
Inhalable particles, Grimm	No.	8.0x10^8 [3.1x10^8 , 1.3x10^9]	9.5x10^7 - 1.8x10^9 9	2.4x10^7 [1.5x10^7 , 3.3x10^7]	1.4x10^7 - 3.4x10^7 4
D_{gae} of particles, Grimm	μm	0.46 [0.43, 0.48]	0.39-0.48 9	0.35 [0.32, 0.39]	0.31 – 0.39 3
Biofilm, OD_{600}, GSP	OD_{600}/m^3	1.00 [0.81, 1.92]	0.31 - 10.0 21	Bd	Bd
TIP, GSP	AUC/m^3	2.8x10^6 [2.1x10^6, 3.6x10^6]	1.5x10^6 - 4.3x10^6 31	1.6x10^6 [3.8x10^5, 2.0x10^6]	1.1x10^6 - 1.9x10^6 4

NA=nutrient agar, GSP= a sampler sampling inhalable particles, ASCI= Andersen six stage sampler. SA= *Staphylococcus* selective agar, TIP= Total Inflammatory Potential, AUC=area under the curve, nm=not measured.

affected significantly by season ($p = 0.0198$), but there were no significant differences between the individual seasons. The concentration of *Staphylococcus* spp. tended to be affected by season with a tendency towards lower concentrations in autumn ($p = 0.075$). The concentration of airborne endotoxin was significantly affected by season ($p = 0.042$) with autumn and summer having the highest concentrations. Significant associations were found between concentrations of *Staphylococcus* spp. ($p = 0.046$) and endotoxin ($p = 0.049$) and inside temperatures with lower *Staphylococcus* spp. and higher endotoxin concentrations during lower temperatures. For bacteria no significant association was found with temperature ($p = 0.16$) and RH ($p = 0.19$). *Staphylococcus* spp. ($p = 0.038$) and endotoxin ($p = 0.014$) concentrations were associated significantly with RH inside the coops with lower *Staphylococcus* spp. and higher endotoxin concentrations during higher RH.

The concentration of bacteria, *Staphylococcus* spp. and endotoxin were not significantly associated with number of pigeons/m^2 during the sampling (ps > 0.05), but for bacteria and *Staphylococcus* spp. there tended to be higher concentrations with more pigeons in total (ps $= 0.09$). The type of pigeon (homing vs fancy) did not affect the concentration of bacteria ($p = 0.79$), *Staphylococcus* spp. ($p = 0.17$), and endotoxin ($p = 0.35$), and there were no significant effect of interactions between season and type of pigeon on the concentrations of the different bioaerosols (ps > 0.05).

The concentration of inhalable particles was measured inside 9 coops in the autumn, and it was between 9.5×10^7 and $1.9 \times 10^9/m^3$ (Table 1), and it correlated significantly with the concentration of *Staphylococcus* spp. ($r = 0.90$, $p < 0.0001$) and endotoxin ($r = 0.86$, $p = 0.0024$). The mass of dust was between 0.18 and 2.06 mg/m^3. The D_{gae} of airborne particles correlated with the number ($r = 0.92$, $p = 0.0021$), and mass ($r = 0.84$, $p = 0.0047$) of airborne particles. The concentration of inhalable particles was greater in the daytime than during the night (Fig. s3).

3.3. Airborne bacteria on particles of different sizes

Bacteria and *Staphylococcus* spp. were present in all six size fraction of the ASCI (Fig. 1). The geometric mean aerodynamic diameter of airborne bacteria sampled on NA was 5.5 ± 0.99 µm, on SA was 4.7 ± 1.1 µm, and on SSI-agar was 4.7 ± 1.9 µm. The D_{gae} of particles with *Staphylococcus* spp. correlated significantly and positively with the RH inside the pigeon coops (Fig. s2).

3.4. Airborne bacterial species as measured using MALDI-TOF MS

In total 39 different bacterial genera and 141 species were identified (Fig. s4), and the species found in highest concentrations on each agar medium are presented in Table 2. *Staphylococcus equorum* was found in all pigeon coops and was also dominating in terms of concentration (GM $= 2700$ cfu/m^3). The concentration of *S. equorum* correlated significantly with temperature ($r = 0.45$ $p = 0.012$), concentration of *S. lentus* ($r = 0.43$, $p = 0.016$), *S. xylosus* ($r = 0.94$, $p < 0.0001$), and *Aerococcus viridans* ($r = 0.44$, $p = 0.032$). The species *S. xylosus* was found in 62% of the coops (GM $= 712$ cfu/m^3) and correlated with concentration of *Ae. viridans* ($r = 0.88$, $p < 0.0001$). The species *Ae. viridans* was found in 43% of all coops (GM $= 677$ cfu/m^3), and its concentration correlated negatively with the RH inside the coops ($r = -0.41$, $p = 0.038$).

Some of the bacteria are classified in risk group 2 (Table 3) of which the pathogens *E. coli*, *S. aureus*, and *Brevundimonas diminuta* were found in several coops. The two first mentioned were found in different particle sizes. Six gram-negative risk group 2 bacteria were identified (Table 3), and the concentration of these bacteria correlated significantly with the absolute humidity ($r = 0.49$, $p = 0.0057$) and temperature ($r = 0.49$, $p = 0.0054$) inside the coops. Of the 40 *S. aureus* isolates none were able to grow MRSA-selective agar.

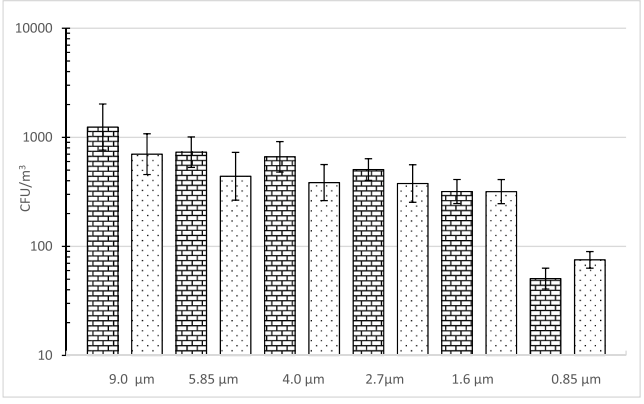


Fig. 1. Concentration of airborne bacteria as measured on nutrient agar (squares; $n = 7$) and on *Staphylococcus* selective agar (dots; $n = 27$) as a function of particle size (D_{ae50} µm).

Table 2
The 15 bacterial species found in highest concentrations on each growing medium, presented as % of total identified species on each medium.

Bacteria	NA	SA	SSI	MRSA-A[x)]
Actinobacter lwofii	0.019		2.95	
Aerococcus viridans	2.51	0.033	1.71	
Arthrobacter arilaitensis	0.282			
Arthrobacter beregeii	0.191			1.03
Bacillus licheniformis		0.298	4.27	0.166
Bacillus megaterium	0.089	0.038		1.03
Bacillus pumilus	0.008	0.033	1.71	0.528
Bacillus simplex	0.111	0.011	0.855	0.0078
Bacillus sp.	0.190			
Bacillus mycoides	0.035			0.528
Brevibacterium aurantiacum	0.460			
Brevundimonas diminuta		0.011		0.616
Corynebacterium stationis		0.153		
Enterococcus mundtii	0.119	1.905		0.047
Erwinia rhapontici	0.008			0.520
Escherichia coli	0.245	0.022	28.4	1.81
Kocuria rosea	0.280			
Micrococcus luteus	0.066	0.873	2.56	
Pantoea agglomerans	0.0038	0.011	2.21	0.016
Pseudomonas antarctica	0.0084			0.520
Pseudomonas brenneri	0.0084			0.520
Pseudomonas extremorientalis	0.0084			0.520
Pseudomonas rhodesiae	0.0084			0.520
Pseudomonas sp.	0.0515			1.039
Sphingobacterium faecium		0.222		
Staphylococcus aureus	0.0028	0.915	1.05	0.0078
Staphylococcus cohnii		0.066	2.10	0.016
Staphylococcus delphini	2.20	3.62		
Staphylococcus equorum	82.2	78.5	25.0	85.3
Staphylococcus kloosii	0.0182	0.155		
Staphylococcus lentus	2.97	4.15	5.98	1.59
Staphylococcus nepalensis	0.271	0.199	2.56	
Staphylococcus saprophyticus	0.0006	0.033	2.56	
Staphylococcus sciuri	0.053	0.195		
Staphylococcus sp.	1.12		1.71	0.520
Staphylococcus succinus	0.328	0.097		
Staphylococcus xylosus	5.34	6.17	7.69	2.60
Species richness[xx)]	89	58	27	44

[x)] Only few and mainly blue colonies identified; [xx)]For all species see **Fig. s4.**

Table 3
Bacterial species found in the pigeon coops which are risk classified, reported as causative agents of occupational diseases, or recently recognized pathogens.

Bacterial species	Number of positive coops out of 31 for MALDI and of 19 for NGS (stages)[1)]	Gram stain	Risk Group 2	Cases of occupational disease	Emerging pathogen	Found using	References
Arthrobacter creatinolyticus	4 (1,2,3,4,5)	-			Yes	M	(Nabal et al., 2020)
Acinetobacter baumannii	11	-		Skin and soft tissue infection		S	(Guerrero et al., 2010)
Acinetobacter lwoffii	5 (1,3,4,5)	-			Yes	M	(Regalado et al., 2009)
Aerococcus viridans	15 (1,2,3,4,5)	+	Yes			M	(Canada, 2018)
Alcaligenes faecalis	1 (4)	-		EAA		M	(Milanowski et al., 1998)
Bacillus cereus	17 (1,2,4,5)	+	Yes	Endophthalmitis		M	(Canada, 2018; Martinez et al., 2007)
Bacillus subtilis	11(2)	+		EAA		M	(Johnson et al., 1980)
Brevundimonas diminuta	14(1)	-			Yes	M	(Ryan, 2018)
Clostridium spp.	19	-	Yes			S	(89/391/EEC, 2000; Canada, 2018)
Corynebacterium minutissimum	1	+	Yes			M	(89/391/EEC, 2000)
Enterococcus faecium	1	+	Yes	Unspecified		M	(Canada, 2018; Haagsma et al., 2012)
Enterococcus hirae	1					M	
Enterococcus mundtii	6					M	
Escherichia coli	14(1,2,3,4,5)	-	Yes	Gastrointestinal		M	(89/391/EEC, 2000; Canada, 2018; Haagsma et al., 2012; O'Brien et al., 2001)
Kocuria spp.	9 (2,5)	+			Yes	M S	(Kandi et al., 2016)
Leclercia adecarboxylata	1	-			Yes	M	(Temesgen et al., 1997)
Legionella sp.	1	-	Yes			S	(89/391/EEC, 2000)
Lelliottia amnigena	2	-			Yes	M	(Capdevila et al., 1998)
Pantoea agglomerans	6 (1,2)	-		EAA		M	(Milanowski et al., 1998)
Prevotella spp.		-	Yes			S	(89/391/EEC, 2000)
Salmonella sp.	1	-	Yes	Gastrointestinal		M	(89/391/EEC, 2000; Haagsma et al., 2012)
Serratia marcescens	1	-	Yes			M	(89/391/EEC, 2000)
Sphingobacterium spiritivorum	18	-		EAA	Yes	S	(Kämpfer et al., 2005; Schröttner et al., 2016)
Staphylococcus aureus	14 (1,2,3,4,5,6)	+	Yes	Skin and mucous membrane		M	(89/391/EEC, 2000; Canada, 2018; Haagsma et al., 2012; Wardyn et al., 2015)
Streptomyces albus	1(1)	+		EAA		M	(Kagen et al., 1981)
Thermoactinomyces sp.	6	+		EAA		M	(Ismail et al., 2006)

M=MALDI-TOF MS and S= Sequencing.[1)]If found using the ASCI the stages positive for the species are mentioned where 1 and 2 will deposit in the upper airways, 3 and 4 in the trachea and bronchioles, and 5 and 6 in the alveoli.

Thirty different bacterial species including 16 *Staphylococcus* species were found on level 6 of the sampler containing particles potentially depositing in the alveoli (Fig. s5).

3.5. Airborne bacterial species as measured using NGS

Using the 16S rRNA gene amplicon sequencing the following 6 species were identified: *Acinetobacter baumannii*, *Butyricicoccus pullicaecorum*, *Peptoclostridium acidaminophilum*, *Sp. spiritivorum*, *Verrucomicrobia bacterium*, and *Weissella jogaejeotgali*. In addition, 180 different genera where identified. The airborne dust was totally dominated by bacteria from the genus *Acinetobacter* which constituted between 3.4 and 65.3% of the ZOTUs. Other dominating genera are *Sphingobacterium* and *Clostridium* (Fig. 2). A total of 16,984 reads across 17 samples (average/sample = 999.0588 ± 473.1841). The species composition was affected significantly by season ($p = 0.017$, ANOSIM statistic $R = 0.139$) but not by type of pigeon ($p = 0.443$) (Fig. 3).

The diversity indices mentioned in Table 4 were not affected by whether samples were taken outside or inside the aviaries (ps > 0.05). Following data were analyzed with random effect of measurement inside vs outside the coops. The biodiversity indices for homing pigeons vs fancy pigeons were not significantly different (ps > 0.05). The Shannon-Wiener index ($p = 0.092$), Chao1 ($p = 0.069$), and richness ($p = 0.037$) were or tended to be higher in summer than in spring (Table 4).

3.6. Inflammatory potential

The total inflammatory potential (TIP) of the samples taken by the GSPs in the coops or outside aviaries was measured (Table 1). The samples induced a low initial ROS production with a mode after 5 min, and no other modes were seen for all samples. Examples are given for coops with homing and fancy pigeons and positive and negative references (Fig. 4). Other examples are given in the supplementary file (Fig. s6). The TIP was associated negatively with RH inside the coops ($p = 0.036$). For four coops with fancy pigeons measurements were done just outside the aviaries and without taken season into account the TIP of these samples was lower than for samples taken inside ($p < 0.0001$).

3.7. Biofilm forming capacity

In total 21 samples were analyzed for biofilm forming capacity. Biofilm measured as OD_{600} was formed in all samples and was between 0.074 and 1.4 OD_{600} (average = 0.22; GM = 0.14), and if normalized for sampled volume and dilution with glycerol between 0.31 and 10.0 OD_{600}/m^3 (Table 1). The biofilm forming capacity correlated significantly with bacteria in general, *Staphylococcus* spp. and numbers of bacteria on SSI agar (Fig. s2). The biofilm forming capacity tended to be higher for samples taken inside than outside the coops ($p = 0.072$), and with higher rh ($p = 0.066$), and there was no association with the number of pigeons in the pigeon coops, types of pigeons, and season (p

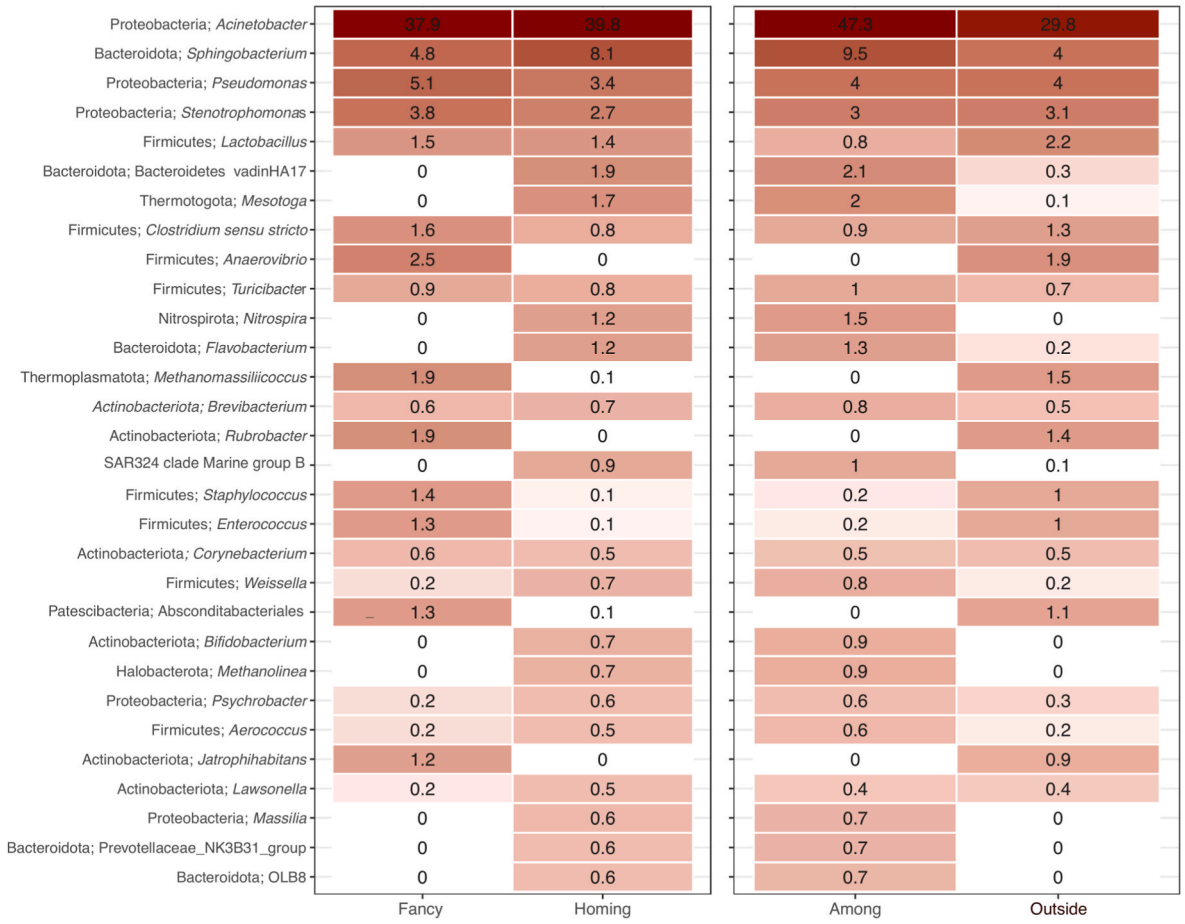


Fig. 2. Heatmap of the 30 most abundant taxa, phylum and genus, observed in the bacterial community measured using NGS.

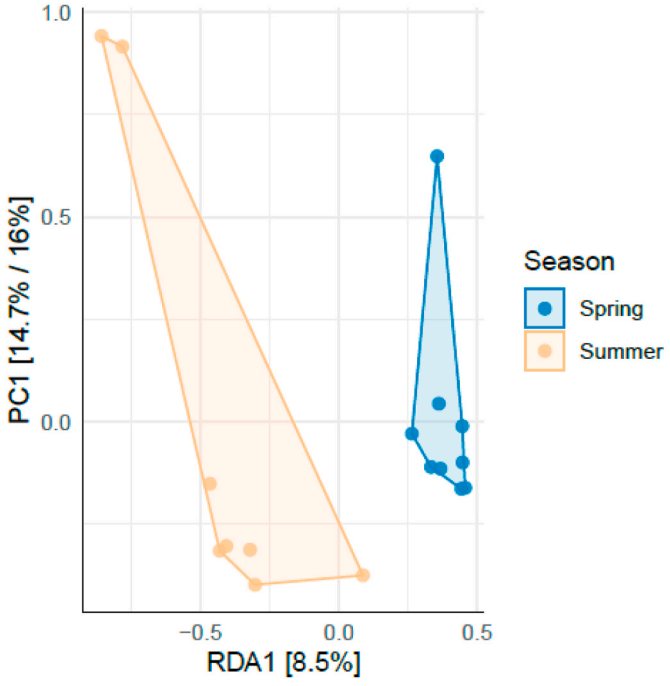


Fig. 3. Constrained redundancy analysis (RDA) plotting of bacterial ZTOUs identified after sequencing constrained by season. Dissimilarity between the clusters was measured by ANOSIM ($p = 0.017$).

Table 4
Biodiversity indices of bacteria (16S rRNA gene – based on ZOTU) in pigeon coops (n = 17); samples are divided into homing and fancy pigeons; samples taken among pigeons and outside the aviaries, and samples taken in spring and summer.

	Among	Outside	Homing	Fancy	Spring	Summer
Shannon-Wiener index (H)[a]	5.86 ± 0.11	6.52 ± 0.36	6.11 ± 0.36	6.29 ± 0.29	5.98 ± 0.33	6.39 ± 0.29
Inverse Simpson[b]	240 ± 50	564 ± 214	385 ± 135	406 ± 85	347 ± 193	444 ± 314
Chao1[c]	848 ± 212	1287 ± 383	1069 ± 338	1029 ± 258	854 ± 291	1281 ± 314
Richness (ZOTU)	783 ± 187	1242 ± 373	1005 ± 330	988 ± 245	810 ± 285	1211 ± 307

[a] Estimator of species richness and evenness: most weight on species richness.
[b] Estimator of species richness and species evenness: most weight on evenness.
[c] Abundance-based estimator of species richness, gives more weight to the low abundance species.

> 0.05).

4. Discussion

Airborne dust from pigeon coops induced only a low and initial ROS production in HL60 cells despite a high content of both bacteria and endotoxin. In addition, the dust contained many bacterial species including several risk classified species and species which have in previous studies in other environments been identified or suggested as causative agents of EAA. This and factors affecting concentrations of

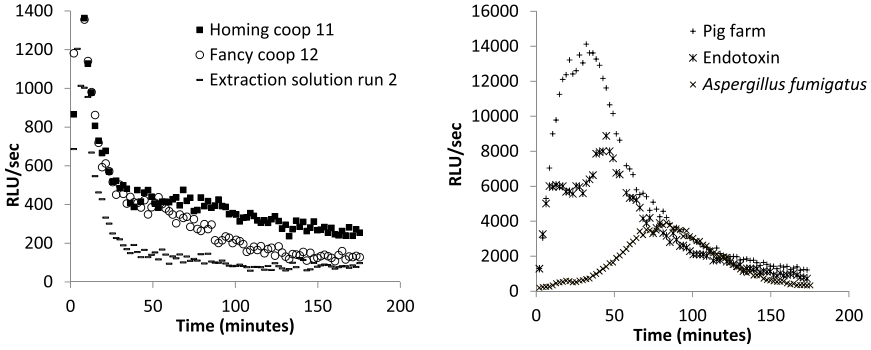


Fig. 4. ROS production of HL60 cells (RLU/second) exposed to dust from homing (coop 5) and fancy pigeon (coop 5), and a negative reference (left); and positive references endotoxin, farm dust and *Aspergillus fumigatus* spores (right) as a function of time. This is raw data, and thus not normalized by time of sampling and the 20 times dilution.

bacteria and endotoxin will in the following be discussed.

4.1. Risk evaluation based on species identity, concentration, and particle size

In this study several species classified in risk group 2 were found (Table 3). Of these *E. coli* and *Salmonella* are mainly known to cause gastrointestinal problems, and both species were found in particle size fractions which may be inhaled or swallowed. However we have not seen any publications about gastrointestinal problems among pigeon breeders –but there is the chance that medical providers might not associate the patients' occupation/hobby with the development of such infections. Both species are gram negative, and thus contain endotoxin which is an inflammogen. The genus *Clostridium* was found in all samples analyzed by NGS and in high reads. Some *Clostridium* species can cause e.g. enteritis and tetanus. Other species classified in group 2 are mainly described as causing infections in immunocompromised persons or in soft skin, e.g. *Corynebacterium minutissimum*, *Enterococcus* spp., and *Se. marcescens*. The species *Ae. viridans* was found in high concentrations, and this bacterium is described as a rare human pathogen (Ezechukwu et al., 2019; Mohan et al., 2017).

Of the species not classified in risk group 2 or higher several of the found species (*Alcaligenes faecalis, Bacillus subtilis*, *Pantoea agglomerans*, *Sp. spiritivorum*, *Streptomyces albus,* and *Thermoactinomyces* sp.) have in previous studies been identified or suggested as causal agents of EAA (Table 3). Therefore it would be relevant to study these species further in relation to the development of pigeon breeder's disease. For the development of EAA it is necessary that the bacteria are present as particles with sizes which can enter the alveoli or bronchioles. Of the species which have previously been described to cause EAA only *Alcaligenes faecalis* was found in this size fraction, but several other bacterial species were found in these size fractions, and could potentially contribute to the development of EEA. However, their ability to do so is at the time of writing, unknown.

For some of the of the bacterial species found in the pigeon coop antibiotic resistance is a recognized problem e.g. *E. coli*, *Salmonella*, *Se. marcescens*, *S. aureus,* and *Streptococcus*, and some species are intrinsically antibiotic resistant e.g. *Brevundimonas diminuta* (Ryan, 2018) and *Leclercia adecarboxylata* (Stock et al., 2004). However, of the 40 *S. aureus* isolates found in this study, none were able to grow on MRSA-selective agar.

4.2. Risk evaluation based on concentrations of endotoxin and bacteria

The concentration of airborne endotoxin in the pigeon coops was up to 745 EU/m^3 with GM of 102 EU/m^3 (n = 28). The outdoor concentration is typically around 1 EU/m^3 (Madsen, 2006). We have found no other studies with measurements of airborne endotoxin in pigeon coops, but it seems to be lower than in poultry houses. Thus, endotoxin concentrations in poultry houses of 4200 EU/m^3 (average, stationary, n not mentioned (nm)) (Bakutis et al., 2004), and in houses with laying hens of 3389 EU/m^3 (median, stationary samples, n = 9) (Schierl et al., 2007) and 234 EU/m^3 (av., n = 2, stationary) (Rimac et al., 2010), and in houses of 50-day-old chickens of 1897 ng/m^3 (personal sample, n = 1) (Lawniczek-Walczyk et al., 2013) have been found. The lower levels found in pigeon coops may be related to the behavior of the pigeons which were mainly sitting quietly during the measurements, and it may also be related to the area per bird. In the pigeon coops 3.9 pigeons were on average present per m^2, in the study mentioned afore 16–17 chickens were present per m^2.

In this study we measured endotoxin using the Limulus kinetic assay, but as another assay, called rFC, is used instead in some studies, we analyzed two samples with that assay to make it possible also to compare levels measured using the rFC assay with results of this study. For these two samples the concentration measured with the rFC assay constituted 48% of what was measured with the Limulus assay. In a study using both assays in different farming environments higher concentrations were in some environments measured using the rFC assay than with the limulus, and in other environments the opposite was found (Thorne et al., 2010). For house dust spiked with endotoxin higher concentrations were also measured with the limulus than with the rFC (Alwis et al., 2006). This may be because the limulus assay is also sensitive to fungi.

In contrast to endotoxin, the concentrations of bacteria was in this study within the ranges of levels found in poultry houses. Thus in poultry houses 466 cfu bacteria/m^3 and 12 g-negative bacteria/m^3 (average, stationary, n nm) (Bakutis et al., 2004) and 1.3 × 10^5 to 2.6 × 10^6 cfu bacteria/m^3 (stationary, n nm) (Lawniczek-Walczyk et al., 2013) have been observed. The concentration of airborne dust was also at the level of what has been found in two poultry farms (Rimac et al., 2010).

There is no official exposure limit for endotoxin and bacteria in Denmark. Studies in occupational epidemiology have shown that exposure to endotoxin increases the likelihood of developing chronic bronchitis, and higher exposure levels lead to higher risk of chronic bronchitis (Basinas et al., 2011; Radon, 2006), but which exposure levels cause chronic bronchitis has not been identified. A study with poultry workers found a significant dose-response relationship between exposure to endotoxin and pulmonary function decrements over a work shift with a significant decrement at 614 EU/m^3 (Donham et al., 2000). Bacteria were not measured in that study. In this study 1 sample exceeded that level. We used stationary samplers, and if we had used personal samplers we would expect to have found higher concentrations due to the closer proximity to the pigeons, and due to the activities of the breeder and the pigeons during e.g. feeding and cleaning of the coops.

4.3. Inflammatory potential

ROS production by granulocytes as a response to exposure to airborne pigeon coop dust was studied for 180 min. In our previous studies a peak in ROS production was typically found after 30 and sometimes again after 60 min – which was also seen in this study for the positive references. Airborne dust from biofuel plants (Timm et al., 2009), waste collection workers (Madsen et al., 2020), and homes (Frankel et al., 2012) have caused a larger ROS production than airborne dust from pigeon coops. For the airborne dust from the pigeon coops a small peak appeared after 5 min of exposure, and then no further reaction was seen. This lack of further ROS production might be because the dust is cytotoxic. Engulfment time is affected by the size and the shape of a particle, and it takes 5.5 ± 0.6 min for a macrophage to engulf an *A. fumigatus* conidium and 1.5 ± 0.9 min to engulf a silica particle of 1.85 μm – the size of a bacterial cell (Paul et al., 2013). In this study phagocytosis may have happened during the first minutes the granulocytes were exposed to airborne dust from pigeon coops.

The absolute dominating cultivable bacterium in the pigeon dust was *S. equorum*. This species is also dominating in pig farm dust (Feld et al., 2018). In this study, pig farm dust was highly inflammogenic compared to pigeon coop dust. Based on NGS data the genus *Acinetobacter* was totally dominating in the air samples, and different *Acinetobacter* species are described to produce a cytotoxic slime (Jin et al., 2011; Obana, 1986), and the *Acinetobacter* species in the pigeon dust may have been cytotoxic.

4.4. Biofilm forming capacity

Biofilm was formed when the pigeon dust was incubated in vitro at 37 °C, and the amount correlated significantly with the concentration of bacteria in general, *Staphylococcus* spp., and numbers of bacteria on SSI-agar. But we do not know which bacteria formed the biofilm. However, isolates of several species found in this study are according to the literature able to form biofilm: e.g. *S. aureus* (White et al., 2020), *S. delphini* (Sudagidan et al., 2012), *S. equorum* (Leroy et al., 2009), and *S. xylosus* (Planchon et al., 2006), *E. coli* (Van Houdt et al., 2005), *Se. marcescens* (Haleva et al., 2020), and *Ae. viridans* (Setiawan et al., 2020). For bacterial species investigated in a previous study, in vitro biofilm-forming capacity correlated well with measurements of biofilm in the upper airways of patients (Bendouah et al., 2006). The ability of microorganisms to form biofilm is a virulence factor as it enables them to form persistent infections and contributes to protecting the bacteria from contact with antimicrobials (Roberts et al., 2010). The biofilm forming capacity of the air samples from the pigeon coops was in the lower end of what has been found for air samples from biowaste sorting plants (Rasmussen et al., 2021).

4.5. Biodiversity

The Shannon-wiener biodiversity index for samples taken among the pigeons were at the levels previously found using the same method for NGS analysis but with personal samplers on people working in cucumber greenhouses (Madsen et al., 2020). The samples taken outside the pigeon houses or aviary had lower concentrations of bacteria and higher Shannon-wiener biodiversity index values, and similarly samples taken using stationary samplers in cucumber greenhouses had lower bacterial concentrations and a higher biodiversity index value (Madsen et al., 2020). The numbers of ZOTUs in this study was in the lower end of what has been found for cucumber greenhouse workers (Madsen et al., 2020). While it is clear that some microorganisms are more inflammogenic than other the importance of the biodiversity of an exposure is still not clear (Madsen et al., 2020). As there are differences in primer sets used for NGS analysis, and on bioinformatic processes for binning reads post-sequencing it is difficult to compare biodiversity indices between studies performed in different research groups.

In this study we made a division of pigeons used for racing called homing pigeons and pigeons mainly used for exhibitions called fancy pigeons. The microbial species compositions as well as the biodiversity indices were not different for these two groups. We do not know whether airways symptoms are more common among homing pigeon breeders than fancy pigeon breeders.

4.6. Bacterial species and the environment

The bacterial species composition in the pigeon coops as measured using MALDI-TOF MS have some similarities with what has previously been found in environments with other birds or animals. Thus the gram-negative bacteria, *E.coli*, *Salmonella* sp., and *Pantoea* sp. found in this study have also been found in poultry houses (Lawniczek-Walczyk et al., 2013), but the other gram-negative species found in the pigeon coops were not identified in samples from the poultry houses. A great overlap in gram-positive species found in this study and species in poultry houses is found (Lawniczek-Walczyk et al., 2013). Only few of the species found in this study were also found in a study in two turkey houses (Fallschissel et al., 2010), but several of the species found in this study have also been found in airborne dust from pig farms (Feld et al., 2018; White et al., 2019). Which and how many species are found in different studies are affected by how many and how large samples have been analyzed as well as the limitations of the identification methods, and therefore the comparisons of environments cannot be complete.

Some of the species of airborne bacteria in the pigeon coops have previously been found in bird droppings. The bacterial genera identified using NGS were dominated by the gram-negative genus *Acinetobacter*. The pathogenic species *A. baumannii* was found in 8 of 17 samples analyzed by NGS and has previously been found in wild bird droppings (Dahiru et al., 2015). *Acinetobacter* spp. (Morakchi et al., 2017) and *Escherichia coli* (Morabito et al., 2001) have also been found in pigeon droppings. The species *S. aureus* has previously been found in pigeon (Faux et al., 1970) and seagull (Cragg et al., 1971) droppings. The species *Enterococcus columbae*, found in most pigeon coops, has been found in pigeon intestines (Devriese et al., 1990), and *Butyricicoccus pullicaecorum* in the cecal of chickens (Eeckhaut et al., 2008). The internal sections of the gastrointestinal tract of birds are dominated by obligate or facultative anaerobes (Grond et al., 2018), and in this study we have found species which are anaerobic and typically found in the gastrointestinal tract. Examples are: *Bacteroides barnesiae*, *Butyricicoccus pullicaecorum, Clostridium, Faecalibacterium prausnitzii*, *Phascolarctobacterium faecium,* and *Turicibacter*.

Bacillus licheniformis, B. megaterium, B. pumilis, and *B. subtilis* found in the air in the pigeon coops have previously been found on plumage of other bird species (Whitaker et al., 2005). Furthermore, several of the bacteria found in this study are able to degrade feathers or to be keratinolytic e.g.: *B. cereus, B. megaterium, B. licheniformis, B. subtilis, Chryseobacterium, Enterococcus faecalis, Flavobacterium* sp ., *Kocuria rosea, Pseudomonas fulva, S. epidermidis, S. haemolyticus, S. hominis, Stenotrophomonas* sp., and *Streptomyces thermoviolaceus* (Gunderson, 2008; Lucas et al., 2003).

4.7. Factors affecting concentrations of dust bacteria, and endotoxin

The concentration of airborne inhalable dust was measured in 10 coops. In a study from Scotland with the same number of pigeons (all homing) per coop a mean concentration of inhalable dust of 0.51 mg/m^3 was measured in periods without activities of the breeder (Morris et al., 1994). This is in the lower end of what is found in this study. In the study by Morris et al. the dust concentration increased during cleaning activities. For endotoxin we found a higher concentration in autumn than in summer, and the same was found for dust in the study by Morris et al. who expect that this is related to the moult in the autumn. For *Staphylococcus* spp. the highest concentrations were found in the summer. Pigeon breeders are in most contact with homing pigeons in August to

November, experience most symptoms of the airways, and have highest levels of antibodies towards pigeon antigens in this period (McSharry, 1983). In this study the bacterial diversity was higher in summer than in spring (no autumn samples were analyzed) and the measured exposures tended to be highest in summer and autumn. In general concentrations of the different bioaerosol components in the different pigeon coops or houses correlated significantly, and this indicates that they likely have the same sources.

For the 31 studied pigeon coops we did not find association between density of coops and concentrations of bioaerosols, but a tendency to higher concentrations of bacteria and *Staphylococcus* spp. In coops with more pigeons. The lack of a significant effect could be related to the anticipated different air change rates of the different pigeon coops. In the study by Morris, an association between dust concentration and number of pigeons was found – but only when measurements were done in absence of the pigeon breeders (Morris et al., 1994).

4.8. The methods and limitations

This study was performed as a cross sectional study in 31 pigeon coops and across pigeon types and seasons general trends concerning the measured bioaerosol components were found as e.g. high concentrations of airborne *Staphylococcus* and *Acinetobacter* and hence we consider the study as representative for pigeon coops in Denmark. We have sampled airborne bacteria using different types of stationary samplers with different advantages. The GSP sampler was selected as it samples the inhalable fraction, has a high sampling efficiency (Kenny et al., 1999), and because the same sample can be used for NGS, biofilm, TIP, and CFU combined with MALDI-TOF, and the same sampler type can be used for sampling dust for gravimetry and endotoxin analysis. The MAS100 was used as it samples directly on the agar plates and hence the loss during extraction is avoided. However, the sampler can only be used for short sampling time, and thus it does not cover a variation over time. By using the ASCI we had the same advantage with sampling directly on the agar and in addition a longer sampling time can be used, and we obtained knowledge about potential deposition of bacteria in the airways. In a future study it would be relevant also to perform personal sampling during the breeder's daily activities in the coops.

For the identification of microorganisms we have used the MALDI-TOF method as well as NGS, and these methods have separately as well as in combination previously shown to be good for this purpose (Decristophoris et al., 2011; Madsen et al., 2019; White et al., 2019). While the MALDI-TOF method is good for identification down to species level the NGS method is good to see systems in the data as many genera are identified. As an example, 6 were identified with species name by NGS while 141 species were identified with species name by MALDI-TOF. On the other hand genera which need specific growth conditions or are sensitive to aerosolisation or sampling were only found by NGS.

For studying the inflammatory potential of the airborne pigeon house dust we used a single cell line HL60 and it may be relevant also to study using other methods. The method was chosen as an immediate effect can measured and then growth of microorganisms are avoided even though the cells are exposed to all the microorganisms from each air sample. The use of the total air sample rather than isolates of selected species is important for this and also for biofilm studies as both the inflammatory potential and biofilm formation can be dependent on the presence of different microorganisms (Haleva et al., 2020; Obana, 1986).

4.9. Perspective

This study was first of all done in relation to potential exposure inside the pigeon coops. However it is also worth noting that coops are often urban and may cause a spread of aerosols from the coops. The coops in this study were all placed in towns, and 20 were placed in private gardens next to the resident while another 11 were placed in a club with several pigeon coops. The concentrations of *Staphylococcus* spp. and bacteria in general were higher outside the aviaries than in reference air; we did not measure concentrations of bacteria and endotoxin in distances from the pigeon coops. The endotoxin concentration (GM) outside the aviaries was 4.7 EU/m^3 which is slightly higher than in normal outdoor air in Denmark. Spread of endotoxin from poultry has previously been shown (Jonges et al., 2015), and pigeon breeder's disease has also been in focus for neighbors to pigeon breeders (Pelikan and Pelikan-Filipek, 1983). A study with *E. coli* in ventilated broiler shed found this bacterium to be robust as an airborne bacterium, and concentrations inside and outside the shed correlated with the concentration in the litter (Chinivasagam et al., 2009). We found *E. coli* inside but not outside the coops. *Sphingobacterium spiritivorum* was observed outside 3 of the 4 aviaries. *Staphylococcus aureus* was found in the air outside one of four aviaries, and *S. equorum*, which is not normally found in outdoor air, was found outside the four coops. It will be relevant to study the further spread of bioaerosols from the coops, but on the other hand, the concentrations outside were considerably and significantly lower than inside the coops.

Antibiotic resistance in *S. aureus* has gained attention internationally through the last two decades. While MRSA in Denmark has mainly been associated with pig farming (Madsen et al., 2018; Sørensen et al., 2020) and is only seldom found in e.g. the home environment (Madsen et al., 2018) it is commonly found in environmental samples in countries with higher prevalence of MRSA in the population as e.g. Portugal (Simoes et al., 2011) and the USA (Gandara et al., 2006). Antibiotic resistant bacteria have previously been found in homing pigeons in Slovakia (Zigo et al., 2017). Therefor we asked the breeders whether the pigeons participated in international competitions, and 84% of the breeders answered yes. To see whether pigeons can be a vector/source of MRSA we measured *S. aureus* and MRSA in the pigeon coops. It was clear that *S. aureus* was found in the air in the coops, but we found no MRSA.

EAA has been described repeatedly among pigeon breeders, and patients with EAA are typically occupied in environments with high and daily exposures. Pigeon breeders typically visit the coops at least twice a day, and this study shows that the concentration of bacteria was in general high and is expected to be higher during e.g. cleaning of the coops. EAA seems to be caused by certain microbial species (Eduard, 2006), therefore it should be noted that the air in the coops contain a wide range of different bacteria including species which have previous caused EAA in other environments. Reduced lung capacity is a long term effect of pigeon breeding (Zacharisen et al., 2002), and since the exposure of the granulocytes to airborne dust from the pigeon coops, in this study, only caused an initial inflammatory response in spite of the presence of bacteria and endotoxin, it is relevant to study whether the pigeon dust is cytotoxicity.

Symptoms of acute attack of EAA resemble the symptoms of Organic Dust Toxic Syndrome (ODTS) (Von Essen et al., 1990). We have previously measured grass seed workers' exposure during development of ODTS, and the dust causing ODTS was in contrast to reference seed dust dominated by several genera including: *Actinetobacter*, *Enterococcus*, *Sphingobacterium*, *Staphylococcus*, and *Stenotrophomonas* (Madsen et al., 2015). These genera were also found dominating in the air in pigeon coops, but several other genera were only dominating in one of the environments. Exposure to endotoxin can also cause development of ODTS, and in one pigeon coop endotoxin concentration was very high (745 EU/m^3). We have found no publication on ODTS among pigeon breeders, but occurrences of ODTS are expected to be underreported in general because symptoms often resemble the flu or other mild illnesses. From this study we cannot conclude that pigeon breeders are at risk of developing ODTS.

5. Conclusions

Bacteria were found on or as particles having the potential to deposit

in different parts of the airways and thus have the potential to initiate an effect on both the upper and lower airways. All air samples contained high concentrations of *S. equorum* (MALDI-TOF data) and *Acinotobacter* (NGS data), and species expected to derive from the plumage as well as the gastrointestinal tract of the pigeons. Thus pigeon breeders will inhale these bacteria on a daily basis.

Bacterial species which are classified in risk group 2 were found in several air samples, but whether they affect the breeders health is not known. Thus, air samples taken among the pigeons contained *Escherichia* sp. of which some were identified as *E. coli*, and hence breeders will also occasionally be exposed to this bacterium. Some isolates of *E. coli* can be infectious and cause e.g. gastrointestinal problems, and therefore attention should also be paid to this species – especially in light of the observation that most breeders were above 50 years and the fact that susceptibility to some infections increase by age (Gardner, 1980).

Different bacterial species which have previously caused development of EAA were also found in the air in pigeon coops, and based on the fact that pigeon breeding is associated with development of EAA it would be relevant also to consider the bacterial exposure of pigeon breeders in the diagnosis of airways symptoms. In addition, airborne endotoxin was found in all pigeon coops in concentrations considerably higher than in reference air, and the daily exposure to endotoxin may also affect the breeders' health.

Correlations were found between several bioaerosol components and also between *Staphylococcus* spp. and number of particles and dust. Therefore a reduction in the amount airborne dust may also reduce the exposure to bacteria. We did not find any association between numbers of pigeons per m^2 and concentrations of bioaerosol components, but a tendency to higher concentration with more pigeons in the coops during sampling. A reduction in the number of pigeons might be associated with a reduced exposure to bacteria and dust. Concerning season, summer and autumn seem to be associated with the highest exposures, and a higher species biodiversity was found in summer than in spring measurements.

The pigeon studied were divided into homing and fancy pigeons, and we did not observe differences in terms of concentrations, species composition as well as biodiversity measures related to this.

Credit author statement

Anne Mette Madsen: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition John Kerr White: Formal analysis, Investigation, Data curation, Visualization, Writing – review & editing Mehmet Emin Keskin: Methodology, Formal analysis, Investigation, Writing – review & editing Jeppe Lund Nielsen: Formal analysis, Data curation, Funding acquisition Kira Tendal: Writing – review & editing Margit Wagtberg Frederiksen: Formal analysis, Investigation, Visualization, Writing – review & editing.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.112404.

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Ethanol extraction in MALDI-TOF MS

Ethanol extraction, also called full extraction, was done for bacteria which were not possible to identify after on-target extraction. Briefly, a small amount of the bacterial colony was suspended in 300 µl sterile MilliQ-water in an Eppendorf tube, vortexed for 1 min., then 900 µl 96% ethanol was added and vortexed for 1 min., centrifuged at 13000×g for 2 min. Then the supernatant was discarded, the pellet was centrifuged at 13000×g for 30 sec. and the pellet left to dry for 5 min at room temperature. Then, a volume of formic acid was added to the sample and the pellet resuspended, and an equal volume of acetonitrile was added to the suspension to neutralize the acid. The sample was centrifuged again at 13.000×g for 2 min, and 1 µl of the supernatant was spotted on the target plate.

Table s1. The pigeon coops and climate during sampling.

Nr. of coops with homing/fancy pigeons	20/11
Average nr. of pigeons per owner (in room of sampling)	65 (39)
Area per pigeon in the coops [range] (n=21), no./m^2	3.9 [1.1-9.1]
Nr. of coops in clubs/private gardens	11/20
Flying competition, national and international	20
Participates in national exhibitions	17
Participates in international exhibitions	7
Buy pigeons from abroad (21 asked)	14 of 21
Average spring temperature inside coops [range] (n=9)	16.9°C [15.3-22.4]
Average summer temperature inside coops [range] (n=8)	24.5°C [20.4-26.6]
Average autumn temperature inside coops [range] (n=13)	13.4°C [3.0-25.0]
Average winter temperature inside coops (n=1)	18.8°C
Correlation between indoor and outdoor temperature	r=0.93, p<0.0001
Average temperature outside (n=21)	19.9°C [12.3-31.1]
Average spring RH inside coops [range] (n=9)	44.6% [32.1-89.3]
Average summer RH inside coops [range] (n=8)	50.8% [41.7-63.9]
Average autumn RH inside coops [range] (n=13)	84.4% [71.0-97.0]
Average winter RH inside coops [range] (n=1)	46.6%
Correlation between indoor and outdoor RH	r=0.92, p<0.0001
Average absolute humidity [range] (n=31)	0.00993kg m^{-3} [0.0050-0.0189]
Average RH outside [range] (n=21)	56% [35.8-94.1]

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Table s2. Methods used for sampling and the following analysis

Method	Pigeon coops A-J	Pigeon coops 1-21
Dates of sampling	13/09/2016 to 29/11/2016	07/05/2019- 20/01/2020
Nr. of samples per season	Autumn: 10	Spring: 9; Summer: 8; Autumn: 3; Winter: 1
GSP, 3.5 lpm	1 h: Polycarbonate filters, NA_{25}+ MALDI-TOF; TIP_{37}. 1 h: Teflon filters, Gravimetry, Endotoxin.	42 to 87 minutes: Polycarbonate filters, $NA_{25+\ 55}$ + MALDI-TOF, TIP_{37}; NGS 16S, $Biofilm_{37}$, Endotoxin.
Andersen sampler, 28.3 lpm	8 min: SA-$agar_{37}$+ MALDI-TOF, 4 min: NA_{25}[1)] + MALDI-TOF, 13 min: MRSA-$agar_{37}$[2)]	8 min: SA-$agar_{37}$+ MALDI-TOF[3)], 8 min: SSI-$agar_{37}$[4)] +MALDI-TOF
MAS100, 100 lpm	-	1 min and 2 min: NA_{25} og NA_{37}, 10 min: MRSA-$agar_{37}$[2)], 10 min: SSI-$agar_{37}$+ MALDI-TOF[4)]
Open petri dish	-	45 min: MRSA-$agar_{37}$
Temperature and RH	Measured for 1 h inside the coops	Measured for 1 h inside and outside the coops
Grimm particle counter	40 min. in 9 coops and 14 h in one coop	-

[1)]3 of 10 samples were not included due to sampling problems or overgrowth of plates.[2)]4 samples taken with ASCI and 20 with MAS100 and blue colonies were identified.[3)]4 plates are not included due to overgrowth.[4)]Samples taken for coops 8-21. NA=Nutrient-agar. The numbers, 25, 37, or 55, in subscript font are incubation temperatures in °C.

A cross sectional study on airborne inhalable microorganisms, endotoxin, and particles in pigeon coops – risk assessment of exposure

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Supplementary files

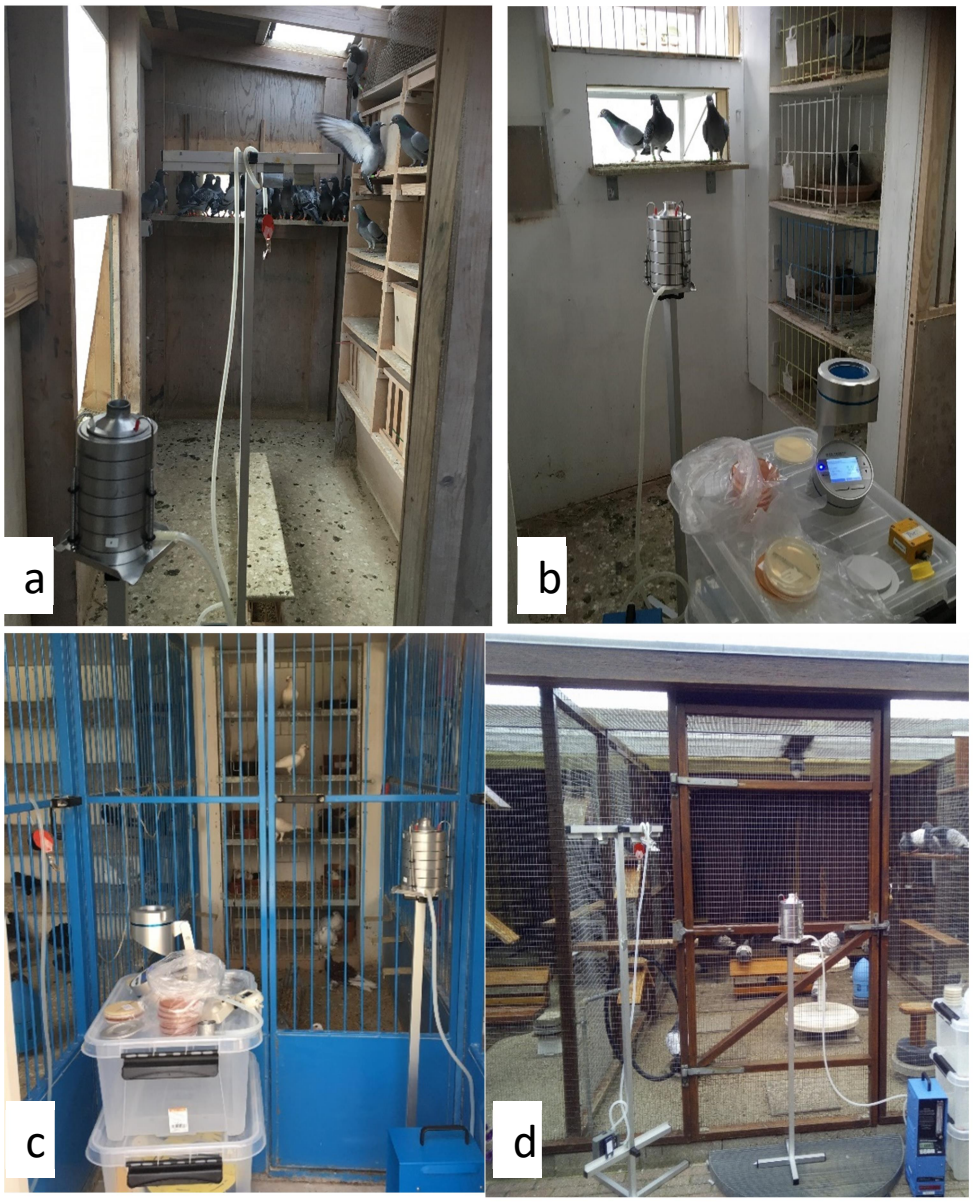


Fig. s1. Examples of places of sampling: Inside homing pigeon coops (ab), Outside the coop of fancy pigeons but inside the pigeon house (c); Outside the pigeon aviary with fancy pigeons – which is outdoors.

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	Temp.													
Rh	**-0.56** **0.0009** **31**													
Bacteria NA GSP	0.26 0.17 31	-0.23 0.25 31	Bact. NA GSP											
Staph. SA ASCI	**0.39** **0.035** **27**	**-0.41** **0.033** **27**	**0.75** **<.0001** **27**	Staph. SA ASCI										
Bacteria NA MAS	**0.55** **0.010** **21**	-0.24 0.28 21	**0.61** **0.004** **21**	**0.98** **<.0001** **17**	Bact. NA MAS									
SSI ASCI	0.30 0.30 14	-0.14 0.63 14	**0.66** **0.015** **14**	**0.69** **0.009** **13**	**0.55** **0.042** **14**	SSI ASCI								
SSI MAS	**0.68** **0.007** **14**	**-0.52** **0.058** **14**	**0.66** **0.013** **14**	**0.69** **0.009** **13**	**0.74** **0.003** **14**	0.51 0.060 14	SSI, MAS							
MRSA agar	-0.25 0.23 24	**0.52** **0.009** **24**	0.24 0.29 24	-0.15 0.53 24	0.12 0.62 21	0.51 0.062 14	0.22 0.44 14	MRSA-agar						
Biofilm GSP	0.06 0.83 21	0.24 0.32 21	**0.69** **0.0008** **21**	**0.63** **0.0049** **17**	0.38 0.087 21	**0.83** **0.0002** **14**	0.36 0.20 14	0.35 0.13 21	Biofilm GSP					
TIP GSP	0.37 0.064 31	**-0.65** **0.004** **31**	0.26 0.21 31	0.19 0.46 27	0.11 0.68 21	0.49 0.086 14	0.24 0.44 14	0.03 0.91 24	0.05 0.82 21	TIP GSP				
Endo. GSP	-0.28 0.14 28	0.40 0.056 28	0.24 0.21 27	**0.53** **0.0048** **27**	**0.53** **0.026** **21**	**0.66** **0.028** **14**	**0.72** **0.014** **14**	0.25 0.24 24	0.43 0.080 21	0.14 0.67 28	End. GSP			
Dust GSP	-0.44 21 10	**0.67** **0.036** **10**	0.44 0.28 10	**0.78** **0.007** **10**	-	-	-	-0.44 0.36 4	-	0.15 0.78 10	**0.92** **0.0002** **10**	Dust GSP		
Particles Grimm	-0.41 0.26 9	0.65 0.060 9	0.67 0.10 9	**0.94** **0.0002** **9**	-	-	-	-0.52 0.36 4	-	-0.36 0.49 9	**0.86** **0.0024** **9**	**0.88** **0.0017** **9**	Particles Grimm	
Dg NA ASCI	0.0091 0.99 7	0.21 0.65 7	-0.25 0.58 7	-0.10 0.83 7	-	-	-	0.55 0.63 4	-	-0.11 0.83 7	-0.27 0.54 7	0.98 0.13 7	-	Dg NA ASCI
Dg SA ASCI	-0.27 0.16 27	**0.38** **0.047** **27**	-0.0027 0.99 27	-0.15 0.75 27	0.13 0.59 17	0.47 0.10 13	0.23 0.45 13	0.15 0.51 21	0.042 0.87 17	-0.26 0.32 17	0.14 0.50 270	0.15 0.850 10	-0.24 0.55 9	0.74 0.056 7

Fig. s2. Correlogram of climate, concentrations of airborne bacteria grown on different media and sampled by different samplers, biofilm formation, the inflammatory potential (TIP) of the aerosols, and geometric mean diameter of particles with bacteria cultivable on NA (nutrient agar) and SA (*Staphylococcus* selective agar). For bacteria on MRSA agar data from the samplers ASCI and MAS100 are pooled. Temp.=temperature inside the pigeon coops, RH=relative humidity inside the pigeon coops, Bact=bacteria, Staph.=*Staphylococcus* on SA, MAS=MAS100 sampler, SSI=bacteria on SSI-agar, MRSA-agar= bacteria on MRSA selective agar, TIP=Total inflammatory potential; end=endotoxin concentration, D_{ga} NA or SA= geometric mean dimeter of airborne bacteria on NA or SA.

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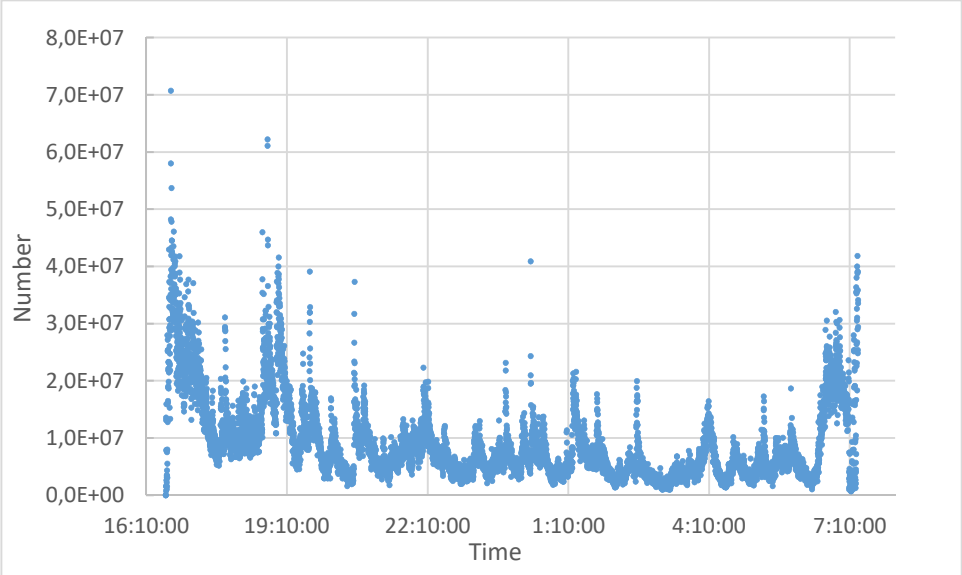


Fig. s3. Concentration of particles (number/m^3) between 1.0 and 6.5 µm as a function of time. Measured in pigeon coop A in November 2018, sunset at 16:15 (when the measurements started) and sunrise at 7:33.

	NA	SA	SSI	MRSA-A
Achromobacter spanius			0.855	
Actinobacter lwofii	0.0191		2.95	
Acinetobacter parvus	0.0014			
Acinetobacter radioresistens			0.855	
Actinobacter sp.	0.0140		0.855	
Aerococcus viridans	2.47	0.0327	1.71	
Alcaligenes faecalis			0.850	
Arthrobacter ardleyensis	0.0825			
Arthrobacter arilaitensis	0.282			
Arthrobacter aurescens	0.0013			
Arthrobacter beregeii	0.191			1.03
Arthrobacter chlorophenolicus	0.0076			
Arthrobacter creatinolyticus	0.0879	0.0794		
Arthrobacter luteolus	0.0095			0.0078
Arthrobacter pascens	0.0013			
Arthrobacter polychromogenes	0.0095			
Arthrobacter psychrolactophilus	0.0189			
Arthrobacter sp.	0.0207			
Arthrobacter stackebrandtii	0.0082			
Arthrobacter sulfonivorans	0.0076			
Arthrobacter tecti	0.0080			
Arthrobacter woluwensis				0.0068
Bacillus altitudinis		0.0034		0.0078
Bacillus amyloliquefaciens ssp plantarum	0.0013			
Bacillus cereus	0.0262			0.198
Bacillus clausii	0.0026			0.0045
Bacillus gibsonii		0.0026		
Bacillus horneckiae	0.0021	0.0980		
Bacillus lentus	0.0099	0.0109		
Bacillus licheniformis	0.0013	0.298	4.27	0.166
Bacillus megaterium	0.0886	0.0377		1.03
Bacillus mojavensis	0.0273	0.0034		
Bacillus muralis	0.0027			
Bacillus mycoides	0.0080			
Bacillus pumilus	0.0083	0.0327	1.71	0.528
Bacillus simplex	0.111	0.0109	0.855	0.0078
Bacillus sonorensis		0.0026		
Bacillus sp.	0.1900			
Bacillus subtilis	0.0297	0.0109		
Bacillus weihenstephanensis	0.0006			0.0078
Bacillus mycoides	0.0353			0.528
Bacillus oleronius				0.0390
Bacillus thuringiensis	0.0360			0.0390
Brevibacterium aurantiacum	0.460			
Brevundimonas diminuta		0.0109		0.616
Corynebacterium ammoniagenes		0.0096		
Corynebacterium aurimucosum		0.0046		
Corynebacterium falsenii				0.0156
Corynebacterium glutamicum	0.0063	0.131		
Corynebacterium minutissimum		0.0046		
Corynebacterium stationis		0.153		
Corynebacterium ammoniagenes		0.0109		
Curtobacterium flaccumfaciens	0.0046			
Curtobacterium albidum	0.0027			

Enterococcus faecium				0.0078
Enterococcus hirae	0.0272			
Enterococcus mundtii	0.119	1.91		0.0468
Erwinia billingae	0.0082			
Erwinia rhapontici	0.0084			0.520
Escherichia sp.			0.194	0.0078
Escherichia coli	0.245	0.0218	28.4	1.81
Exiguobacterium sp.		0.0149		
Jeotgalicoccus halotolerans		0.131		
Kocuria carniphilia	0.0298			
Kocuria palustris		0.0045		
Kocuria rosea	0.280			
Kocuria sp.	0.0025			
Leclercia adecarboxylata			0.194	
Lelliottia amnigena			0.387	
Lysinibacillus boronitolerans		0.0436		
Microbacterium aerolatum				0.0098
Microbacterium maritypicum	0.0046			
Microbacterium paraoxydans				0.0390
Microbacterium phyllosphaerae	0.0006			
Microbacterium testaceum				0.0196
Micrococcus luteus	0.0657	0.87	2.56	
Oceanobacillus profundus		0.0089		0.0156
Paenibacillus amylolyticus	0.0044			
Paenibacillus illinoisensis				0.0078
Pantoea agglomerans	0.0038	0.0109	2.21	0.0156
Pantoea sp.	0.0173			
Providencia rettgeri			0.194	
Pseudoclavibacter helvolus	0.0013			
Pseudomonas antarctica	0.0084			0.520
Pseudomonas avellanae	0.0173			
Pseudomonas brenneri	0.0084			0.520
Pseudomonas clororaphis	0.0084			
Pseudomonas corrugata	0.0250			
Pseudomonas extremorientalis	0.0084			0.520
Pseudomonas frederiksbergensis		0.0109		
Pseudomonas fulva	0.0193			
Pseudomonas koreensis	0.0114		0.855	
Pseudomonas rhizosphaerae	0.0084			
Pseudomonas rhodesiae	0.0084			0.520
Pseudomonas sp.	0.0515			1.04
Pseudomonas fulva		0.0109		
Psychobacillus psychrodurans	0.0006			
Rothia nasimurium	0.0025	0.0109		
Salmonella sp.			0.580	
Serratia marcescens				0.0078
Solibacillus silvestris	0.0173			
Sphingobacterium faecium		0.222		
Sphingomonas aerolata	0.0080			
Sphingomonas faeni	0.0013			
Staphylococcus arlettae		0.0218		
Staphylococcus aureus	0.0028	0.915	1.05	0.0078
Staphylococcus capitis		0.0056		
Staphylococcus caprae		0.0080		
Staphylococcus chromogenes	0.0076	0.0218		
Staphylococcus cohnii		0.0655	2.10	0.0156
Staphylococcus delphini	2.20	3.62		
Staphylococcus epidermidis	0.0025	0.0545		
Staphylococcus equorum	82.2	78.5	25.0	85.3
Staphylococcus fleurettii	0.0012	0.0436	0.855	0.0310

Staphylococcus haemolyticus		0.349		0.0156
Staphylococcus hominis		0.0109		
Staphylococcus hyicus		0.0109		
Staphylococcus intermedius		0.0218		
Staphylococcus kloosii	0.0182	0.155		
Staphylococcus lentus	2.97	4.15	5.98	1.59
Staphylococcus nepalensis	0.271	0.199	2.56	
Staphylococcus pasteuri		0.0436		
Staphylococcus saprophyticus	0.0006	0.0327	2.56	
Staphylococcus sciuri	0.0534	0.195		
Staphylococcus sp.	1.12		1.71	0.520
Staphylococcus stationis		0.0089		
Staphylococcus succinus	0.328	0.0973		
Staphylococcus vitulinus	0.0698			
Staphylococcus warneri	0.0006	0.0005		
Staphylococcus xylosus	5.34	6.17	7.69	2.60
Stenotrophomonas rhizophilia	0.0082			
Streptomyces albus	0.0006			0.0156
Streptomyces avidinii	0.0095			
Streptomyces badius	0.0623	1.15		
Streptomyces griseus	0.0012			
Streptomyces scabiei				0.0156
Streptomyces thermoviolaceus	0.0006			
Streptomyces violaceoruber				0.0156
Thermoactinomyces sp.	0.0012			
Viridibacillus neidei	0.0051			

Fig. s4. Heat map of bacterial species in 31 pigeon coops presented as % of total on each agar medium.

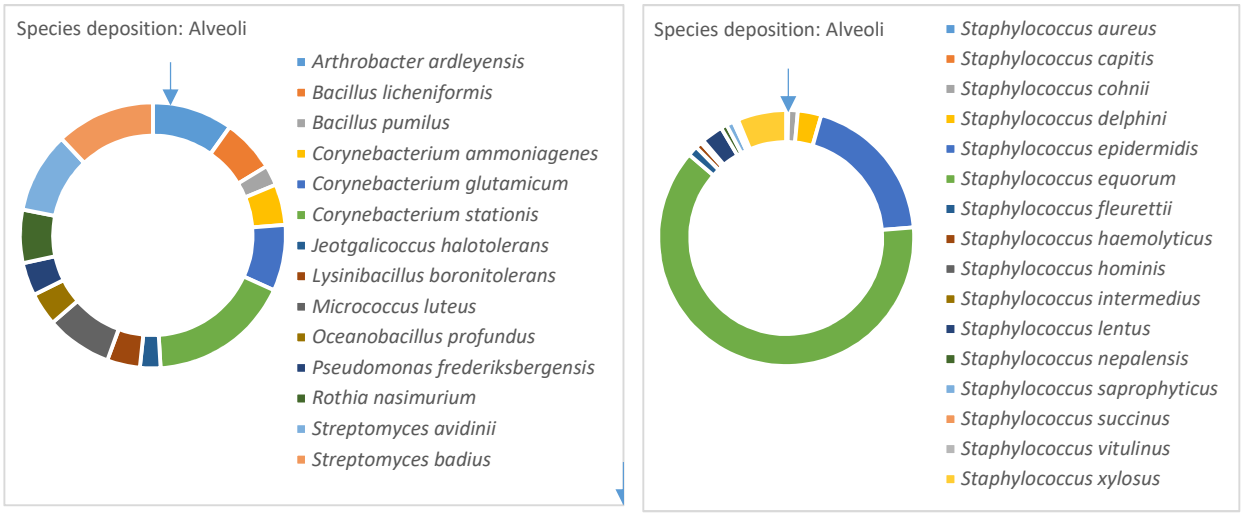


Fig. s5. Bacterial species potentially depositing in the alveoli presented as % out of total bacteria in the size fraction divided into non-*Staphylococcus* (left) and *Staphylococcus* (right) species. The blue arrows indicate the first mentioned bacterium.

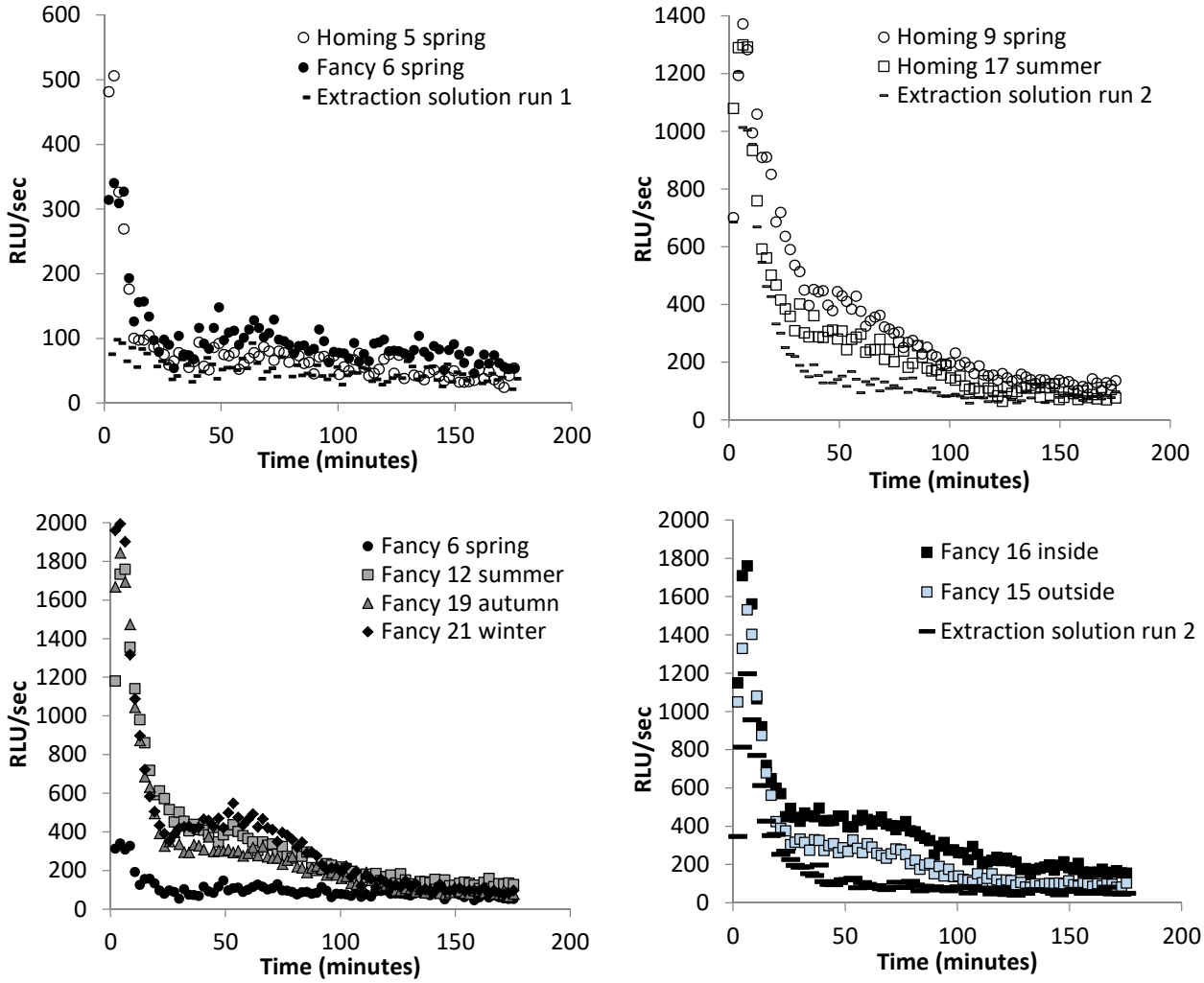


Fig. s6. ROS production of HL60 cells (RLU/second) exposed to dust from homing (coop 5) and fancy pigeon (coop 6) in the spring, and a negative reference (top left); homing pigeons in spring (coop 9) and summer (coop 17), and a negative reference (top right); fancy pigeons in spring (coop 6, run 1), summer (coop 12, run 2), autumn (coop 19, run 2) and winter (coop 21, run 2) (bottom left), and inside (coop 16) and outside (coop 15) fancy pigeon coops and a negative reference (bottom right). This is raw data, and thus not normalized by time of sampling and the 20 times dilution. The reactivity of the cells can differ by time, and therefore the number of the run is given.