BIOAEROSOLS IN EASTERN CANADIAN DAIRY BARNS USING TIE- AND FREE-STALL HOUSING

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Research

HIGHLIGHTS

- Building characteristics (ventilation and animal density) seem to have an important effect on air quality.
- Concentration in the air seems to be influenced by amounts in the bedding for certain air quality indicators.
- Dust concentrations were below OSHA threshold, while some barns exceeded DECOS threshold for endotoxins.
- This study gives new data into the biological components in the air of dairy barns (bacteria, molds, endotoxins, etc.)
- Free-stall or tie-stall housing may not be linked with poorer air quality.

ABSTRACT. *Alternative farming methods make it possible to satisfy public demands for animal welfare while preserving production efficiency, with free housing in dairy farms being an example. The increased movement of cows may have a negative impact on air quality and the presence of etiological agents, increasing the prevalence of lung disease in workers. Free-stall farms, however, are more spacious and modern than tie-stall farms. This study characterizes air quality in free-stall and tie-stall farms (dust, total bacteria, Penicillium/Aspergillus, archaea, and endotoxins). It also focuses on detecting airborne etiological agents and indicators of fecal contamination, as well as assessing the effect of environmental factors on air quality. Five farms of each type (free housing and tie housing) using straw bedding material and equipped with mechanical ventilation were visited. Sampling visits were conducted in winter with no activity (e.g., bedding spreading) in buildings. Dust was evaluated using the DustTrakTM DRX Aerosol Monitor, and bioaerosols were sampled in triplicates for 10 minutes using the SASS®3100 Dry Air Sampler. Finally, soiled bedding was collected throughout the barn. No type of housing seems to be linked with poorer air quality, but some air quality indicators stood out in some outliers. The most recently designed free-housing buildings and the greater air volume may have played a role in the absence of detected differences. Escherichia coli, Enterococcus spp., Clostridium perfringens, Aspergillus fumigatus, Staphylococcus aureus, Saccharopolyspora rectivirgula, Coxiella burnetii, and Klebsiella pneumoniae were detected in high concentrations in both types of buildings. Soiled bedding concentrations, ventilation rates, and animal density seemed to have a significance on air quality in dairy barns.*

Keywords. *Air Quality, Bioaerosols, Housing, Occupational exposure.*

Respiratory problems among dairy farmers are associated with inhaling significant concentrations of organic dust particles (bioaerosols) in their working environment (Reynolds et al., 2013). Bioaerosols are airborne suspensions of particles smaller than 200 microns containing biological agents (e.g., bacteria, molds, endotoxins) (Hinds, 1999). Particles can be aerosolized from several sources (e.g., bedding, manure, cows, workers, feed) (Thaon et al., 2011) and during activities such as milking, bedding spreading, and cow movement. These particles can reach the respiratory system of workers and cause infectious or non-infectious diseases. For example, inhalation of bioaerosols from dairy production was linked to the development of hypersensitivity pneumonitis (or extrinsic allergic alveolitis) such as farmers' lung disease (Cormier et al., 1985; Duchaine et al., 1999; Reynolds et al., 2013). Associations between the duration of exposure to bioaerosols and the reduction of lung function in dairy farmers have been revealed by past studies, with cases of chronic bronchitis, asthma, and hypersensitivity pneumonitis (Melbostad et al., 1997; Reynolds et al., 2013; Stoleski et al., 2019).

Few exposure limits are established for the many biological agents found in the air of dairy barns. Total airborne dust rarely exceeds the permissible exposure limit of 10 mg m^{-3}

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of the Occupational Safety and Health Administration (OSHA), with previous published values between 0.2 and 9.5 mg m^{-3} (Louhelainen et al., 1987; Virtanen et al., 1988; Basinas et al., 2015). Airborne endotoxins, a Gram-negative bacteria cell wall component linked with allergic respiratory symptoms (e.g., asthma, lung function decline) (Basinas et al., 2012), sometimes far exceeded the exposure limit value of 90 endotoxin units (EU) per cubic meter recommended by the Dutch Expert Committee on Occupational Safety (DECOS) (Health Council of the Netherlands, 2010). Airborne endotoxins were already found at concentrations between 21 and 34,800 EU m^{-3} in dairy productions (Kullman et al., 1998; Samadi et al., 2012, Basinas et al., 2015).

Zoonotic agents may also be present in dairy production (Tomley and Shirley, 2009; Palomares Velosa et al., 2021; Guo et al., 2022). Transmission to dairy producers occurs primarily through contact (direct or indirect) with animals, soiled bedding, and inhalation of bioaerosols. Unfortunately, no exposure limit is established for the different zoonotic agents due to the difficulties behind establishing new exposure limit value (ELV). This is mainly due to the variability of the response between individuals, the diversity of microorganisms in bioaerosols and their synergistic effects, as well as their adaptation to the environment. Due to the presence of manure, fecal bacteria can be found in dairy farms, but information about their presence in the air remains scarce. These include *Escherichia coli, Enterococcus* spp*.,* and *Clostridium perfringens*, which can be linked with food poisoning when consuming highly contaminated dairy products (Lambertini et al., 2015, Dapkevicius et al., 2021,; Santos et al., 2022). *Clostridium perfringens* has also been linked with necro-hemorrhagic enteritis, with a low morbidity rate but mortality rate of close to 100% (Lebrun et al., 2010). Molds like *Aspergillus fumigatus*, responsible for aspergillosis (CDC, 2022), are frequently retrieved from straw bedding of dairy barns (Shadmi et al., 1974; Whitlow and Hagler, 2008) and were also previously found in the air of dairy barns (Mbareche et al., 2019). Other agents, such as archaea responsible for the inflammatory response in the lungs of dairy farmers, were previously found in the air of dairy barns (Blais Lecours et al., 2011; Blais Lecours et al., 2012). Finally, *Saccharopolyspora rectivirgula*, an actinomycete found in damp straw, the cause of some cases of farmers' lungs (Pepys et al., 1990), was previously found in the air of Quebec dairy productions (Duchaine et al., 1999; Blais Lecours et al., 2012).

Some recurrent zoonotic agents in cows can lead to diseases such as mastitis, an infection of the cow's udder changing milk production and consistency. Mastitis can be contagious, such as those caused by *Staphylococcus aureus* (Dufour et al., 2012)*,* or environmental, such as *Klebsiella pneumoniae* (Cheng and Han, 2020). Even though these bacteria have been previously found in dairy barns, they don't all represent the same risk. Virulence factors significantly influence the prevalence of mastitis in cow herds, as some specific factors like hemolysin are linked with a higher prevalence (Pichette-Jolette et al., 2019). In humans, *S. aureus* can cause opportunistic infections, pneumonia, and even toxic shock syndrome (Lin and Peterson, 2010), while *K. pneumoniae* may be responsible for pneumonia, especially in immunocompromised individuals (Paczosa and Mecsas, 2016). Their presence in the air of dairy barns has been previously reported with culture methods (Abd-Elall et al., 2009). *Listeria monocytogenes*, linked with cases of listeriosis in cattle and humans, can also be found inside dairy barns (Cooper and Walker, 1998; Vilar et al., 2007). Moreover, *Coxiella burnetii*, an intracellular bacterial pathogen responsible for coxiellosis in cattle, can cause Q fever (query fever) in dairy workers (Robi et al., 2023). The primary transmission route of this pathogenic agent is by inhalation of bioaerosols from animal sources, mainly cattle amniotic fluid (Woldehiwet, 2004, Eldin et al., 2017). This bacterium has been previously detected in many Quebec dairy barns (Turcotte et al., 2021). The presence of these zoonotic agents in the air of dairy barns remains vastly uncharacterized.

In the province of Quebec (Eastern Canada), dairy production is the main agricultural activity in terms of monetary receipts (Ministère de l'Agriculture, des Pêcheries et de l'Alimentation, 2021), with 4,877 dairy farms providing 36% of Canadian milk (Government of Quebec, 2019). A significant aspect of sustainable development of the dairy industry is animal welfare, since consumers demand humane care, transport, and slaughter of the animals (Cornish et al., 2016). Next-generation farms aim to increase production efficiency while implementing procedures to improve the health and well-being of livestock (Thornton, 2010). The use of a more comfortable substrate for bedding, greater freedom of movement, precision feeding, and disease control are examples of changes undertaken in several types of animal production (cattle, pigs, laying hens, goats, broiler chickens, and sheep) (Fraser et al., 2013). With the renovation or construction of new dairy productions according to the new code of practice (NFACC, 2023), some Quebec producers no longer use tie stalls, as free housing can be linked to better udder health, lower occurrence of diseases and lameness, and sometimes, better milk production over time (Von Keyserlingk et al., 2009; Beaver et al., 2021). Although beneficial to animals, more space and freedom of movement for animals may provide suitable conditions for aerosolizing dust and other biological agents (e.g., bacteria, molds), which may negatively impact air quality and human health.

However, modern barn conception and management practices (dimensions, ventilation rates, waste management, milking systems) may mitigate the production of dust and bioaerosols and may help maintain adequate air quality. This mitigation could lead to an absence of differences in air quality between the two types of barns. As data on the effect of free housing for dairy cows on air quality remains scarce and the presence of airborne etiological agents remains vastly uncharacterized, more studies are needed to assess air quality in these new environments.

The present study aimed to characterize the air quality of five dairy barns using tie stalls and five others using free stalls. Concentrations of airborne dust particles (total, PM10 [Particulate matter 10], PM4, PM2.5, and PM1), gases (carbon dioxide, ammonia, and nitrous oxide), total bacteria, *Penicillium/Aspergillus* (Pen/Asp), archaea, and endotoxins were assessed for both types of farms. Statistical comparisons were also made to detect any trend that would help interpretations. Fecal indicators and etiological agents were

also detected and quantified to characterize the bioaerosols of dairy production. Finally, the present study aimed to evaluate the effects of environmental factors (ventilation, animal density, temperature, relative humidity) on bioaerosols in the visited dairy production.

MATERIALS AND METHODS

RECRUITMENT OF *DAIRY FARMS*

Ten dairy barns (n=10) (5 using tie stalls and 5 using free stalls) using mechanical ventilation and straw as bedding material were recruited for the sampling periods (fall and winter 2021 and 2022) by contacting dairy farms around the central region of Quebec (Eastern Canada) and in the region of Chaudière-Appalaches. A questionnaire was then answered by the owners to verify eligibility. Sampling periods took place during the cold season (October to March). Three farms of each housing type were visited in 2021; the last four were visited in 2022 (November to December). Since mechanical ventilation is drastically reduced during the winter, higher concentrations of bioaerosols and poorer air quality were expected. The use of straw as bedding material was a selection criterion for dairy farms' recruitment due to its widespread use and potential for particle emissions (Samadi et al., 2012; Lactanet, 2021). A questionnaire was filled out with the help of dairy producers to gather information about the producers' activities and characteristics of the barn (e.g., dimensions, number of cows, milking system, waste management).

Temperature, Relative Humidity, and Ventilation Rate

Humidity and temperature were measured inside the visited barns, close to each fan in operation, and at various locations in the building. These locations were established according to how the air travels from the entrance to the exit and according to the available space in the buildings (workers' spaces). Two to three measurements per location were made using a telescopic multifunction probe thermos-hygro-anemometer (model VT 210 M, Kimo Electronics Pvt. Ltd., 5 Magnum Opus, Vakola, Santacruz [E], Mumbai 400055, India.), which records temperature, humidity, and air velocity values every second and calculates the averages automatically. In most of the barns visited, three measures were taken at each working fan and then averaged. For some barns, the number of working fans was higher, and the barn was bigger. To make sure all measurements were taken during the bioaerosol sampling time window, the number of measurements per location was reduced to two and averaged. The heights of these measurements were at an approximate height at which workers inhale. External environmental conditions (temperature and humidity) were also measured close to the air inlets.

The airflow rate was assessed by performing a traverse, during which the air velocity of the working fans' cross-sections was carefully measured at several points across the perimeter of the fan. Six sections were established for each fan diameter, and 30 values were recorded for each fan section. We determined the measuring period of the fans according to their operation time during sampling, since the operation time of fans in winter is short and not continuous. Then, the airflow rate was calculated by using:

$$q_f = v \times A \times \Omega \quad (1)$$

where q_f is the airflow rate of each fan (m^3 of moist air h^{-1}), v is the average air velocity ($m.h^{-1}$), A is the fan section (m^2) and Ω is the duty cycle expressed in percentage (%) of the time during which the fan f was active (Rosa et al., 2019). The ventilation rate (VR) (m^3 of moist air h^{-1}) of each farm is then obtained through the sum of the airflow rates of all measured fans (eq. 2), which represents the volume of air entering or leaving the building in a given amount of time.

$$VR = \sum_{f=1}^{n} q_n \quad (2)$$

Evaluating ventilation rates with direct measurements (standard protocol) is mostly done for longer periods of measurements and for single buildings, as well as with the use of tunnels to mitigate turbulence. Since punctual measurements were taken in different buildings, it is hard to use a standard method. The protocol used in this study was developed and validated by comparing punctual and direct air flow measurements in livestock buildings with indirect measurements (CO_2 balance and thermic balance).

GAS CONCENTRATIONS

The concentration of carbon dioxide (CO_2), ammonia (NH_3), and nitrous oxide (N_2O) was measured by using an infrared gas analyzer (FTIR model DX4040, Gasmet) in parallel to the temperature and humidity measurements. These gases can originate from the metabolism of the animals or microorganisms present in the environment and are common air contaminants (Government of Canada, 2017; Miller et al., 2010). Two to three measurements of gas concentration were made at each measuring location during the bioaerosols sampling periods and mean concentrations were calculated for each barn.

MEASUREMENTS OF AIRBORNE DUST

Dust particles were measured simultaneously with bioaerosols sampling in the workers' spaces as close as possible to the central areas of the building, away from working fans, and in the absence of workers' activity (e.g., feed distribution). Three measurements of 10 min were made at a height of 1 m above ground using an optical particle counter, the DustTrak™ DRX Aerosol Monitor (3 L min^{-1}, Model 8534, TSI, Shoreview, Minn.). A reading (data log) of one concentration value per second was done for the 10-min duration. Concentrations of the different particle size fractions (total dust, PM1, PM2.5, PM4, and PM10) were then averaged and expressed in milligrams per cubic meter (mg m^{-3}) for each barn. PM10 stands for "Particulate Matter 10" and represents a fraction of dust particles having a 10 microns aerodynamic diameter or less. Total dust includes particles having a diameter between 0.1 and 15 µm.

SAMPLING OF BIOAEROSOLS

Bioaerosol sampling was performed simultaneously with dust measurements. Baseline concentrations of bioaerosols were evaluated by sequentially collecting three samples for 10 minutes each using a SASS® 3100 Dry Air Sampler with

an electrostatic filter (300 L min^{-1}, Research International, Monroe, Wash.).

In addition, 30-minute samples (1 for each dairy farm) were collected using the SASS® 3100 Dry Air Sampler for the detection of potentially low-concentrated airborne etiological agents. Indoor field blanks were used as negative controls. Finally, bioaerosol samples taken outside with a SASS® 3100 Dry Air Sampler were used as outdoor controls and analyzed along with bioaerosol samples from inside dairy barns.

Sampling of Soiled Bedding Material

For the first six barns, soiled bedding (at least 25 mg) was collected from at least three different locations within each visited dairy barn using sterile bags. For the last four barns, soiled bedding was collected from multiple locations throughout the barns to form composite samples to have a better representation of the whole barn and to increase the chances of finding rare events. A field blank was also carried out using an empty sterile bag that was treated like other bags but didn't contain any soiled bedding.

Sample Processing

Bioaerosols were extracted from the SASS®3100 filters using 7 ml of phosphate-buffered solution (138 mM sodium chloride, 2.7 mM potassium chloride, 0.05% Triton X-100, <0.1% sodium azide, 10 mM sodium phosphate, pH 7.4) and the SASS® 3010 Particle Extractor (Research International, Monroe, WA, USA). Extracts were then centrifuged at 21,000 RCF for 10 min, and the supernatant was removed. Pellets were kept at -20° C until DNA extraction. Extracts of the 30-minute samples from the SASS®3100 were filtered on a 0.22 µm polycarbonate membrane (Isopore™, Ireland, Tullagreen, Carrigtwohill, Co. Cork) [designated as "concentrated air samples"]. The filters were kept at -20°C until DNA extraction. Total DNA was extracted using the DNeasy® PowerLyzer® PowerSoil® DNA Kit (QIAGEN, Mississauga, ON, Canada) and kept at -20°C until the qPCR analyses.

For soiled bedding samples, the Fisherbrand™ Triple Mix Paddle Blender (Fisher Scientific, Hampton, N.H.), was used to mix 25 g of soiled litter samples into 200 mL of a phosphate-buffered saline solution (PBS 1×) containing 0.05% Tween 20. The aliquots of 1 ml were centrifuged, the supernatant removed, and the pellets were kept at -20° C until DNA extraction. To determine the percentage of dry matter of each sample, 25 g of soiled bedding were dehydrated in a Thelco® Laboratory Oven (Precision Scientific, Chennai, Teynampet, Anna Salai) at 105°C for 72 h. Weight was measured before and after the drying to obtain the percentage of dry matter.

Quantification of Bacteria, Molds, and Specific Etiological Agents

Quantitative polymerase chain reaction (qPCR) was performed using the CFX96 or the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, Calif.). Table S1 lists the microorganisms, and the sequences of primers and probes used for the present study. For all microorganisms to be quantified, a plasmid containing the qPCR-targeted sequence was used to construct a standard curve. All synthetic plasmids, primers, and probes were purchased from Integrated DNA Technologies (Coralville, Iowa). A non-template control and a 10-fold serial dilution of a standard curve (between 1 and 1×10^6 copies of a plasmid) were included in all qPCR run. Mean concentrations were expressed in copies per cubic meter of air (copies m^{-3}) or copies per gram of dry matter (copies g^{-1}) for each barn. The targeted microorganisms include general air quality indicators (total bacteria, *Penicillium/Aspergillus,* and archaea*)*, fecal indicators (*E. coli*, *Enterococcus* spp., *L. monocytogenes,* and *C. perfringens*), and some etiological agents (*S. rectivirgula*, *C. burnetii, A.fumigatus, S. aureus,* and *K. pneumoniae*).

Quantification of Endotoxins

Airborne endotoxins were quantified from the 10-minute SASS® 3100 air samples using the Kinetic-QCL™ Chromogenic Limulus Amebocyte Lysate Assay (LAL) kit (LONZA, Walkersville, Md.). The standard curve consists of a serial dilution of standard endotoxins (between 0.005 and 5.0 EU mL^{-1}). Reads were made using the Synergy H1 Hybrid Microplate Reader (Biotek, Winooski, Vt.). Mean endotoxin concentrations were expressed in endotoxin units per cubic meter of air (EU m^{-3}).

Statistical Analyses

Data analyses were performed using R software (version 4.2.3) and Microsoft Excel. Since the variables were quantitative and not normally distributed, non-parametric tests were used with medians. For reach barn, all samplings were pooled to obtain medians of all air quality indicators per barn. Statistical comparisons of medians between tie-stall barns and free-stall barns were made using Kruskal-Wallis tests, with an α threshold of 0.05 for statistical significance. Even though the main objective of the study is to characterize, comparisons were made to detect any trends between both types of barns and as proof of concept. A non-metric multidimensional scale analysis (NMDS) using Gower distance was performed to visualize the association of environmental factors with bioaerosols, as well as potential outliers in the replicates. To obtain a good fit of the model, only the air quality indicators that had enough positive replicates were included in the NMDS model. The model is then given a score (stress value), which should be below 0.1 to be considered fair or below 0.05 to be considered good. Scaling was measured using the Gower distance between the airborne data, bedding concentrations, and building characteristics. The model was also used to highlight potential outliers, which would be barns that stand out for specific variables. A Spearman correlation matrix was made to assess the potential one-on-one links between the different data sets (bioaerosols and environmental factors).

Results

Barn Characteristics

Since this study focuses on air quality, animal density was expressed in cows m-3. Animal density was significantly higher in tie-stall barns ($p = 0.009$) (table 1). Milking in tie-

Table 1. Building characteristics and environmental conditions in the visited dairy barns.

Parameters	Tie-Stall Barns	Free-Stall Barns
Animal density (cows m^{-3})[a]	0.041-0.057	0.019-0.037
No. of milkings per day	2	2-3
Temperature inside the barn (°C)	7.6-12.8	5.8-9.6
Relative humidity inside the barn (%)	56.7-70.9	60.1-81.1
Ventilation rate (m^{-3} of moist air h^{-1})	2.33-4.83	0.78-3.21

[a] Animal density was obtained by dividing the number of cows by the approximate volume of air in the building ($p<0.05$, Kruskal-Wallis).

stall barns was usually done twice a day. In free-stall barns, cows were entering a milking robot, two to three times a day. The temperature ranged between 7.6°C and 12.8°C for tie-stall barns and between 5.8 and 9.6°C in free-stall barns. Relative humidity varied between 56.7% and 70.9% in tie-stall barns and between 60.1% and 81.1%. As for ventilation rates, values raged between 2.33 and 4.83 m^{-3} of moist air h^{-1} for tie-stall barns and between 0.78 and 3.21 m^{-3} of moist air h^{-1} No significant difference was found for temperature, relative humidity, or ventilation rates using a Kruskal-Wallis test ($p > 0.05$).

Concentrations of Gases

The CO_2 values varied between 2,177 and 3,026 ppm for tie stalls and between 1,186 and 2,989 ppm for free stalls (fig. 1). Ammonia concentrations ranged from 1.85 to 4.92 ppm for tie stalls and from 3.92 to 9.36 ppm for free stalls. Finally, N_2O values were between 0.26 and 0.34 ppm in tie stalls, whereas free stalls had concentrations between 0.28 and 0.36 ppm. No statistically significant differences were observed for gas concentrations between the two types of dairy farms ($p> 0.05$, Kruskal-Wallis test).

Concentrations of Airborne Dust

For the finest fractions of dust particles (PM1, PM2.5, and PM4), concentrations between 0.0182 and 0.086 mg m^{-3} were observed for tie-stall farms and between 0.009 and 0.069 mg m^{-3} for free-stall farms (fig. 2). PM10

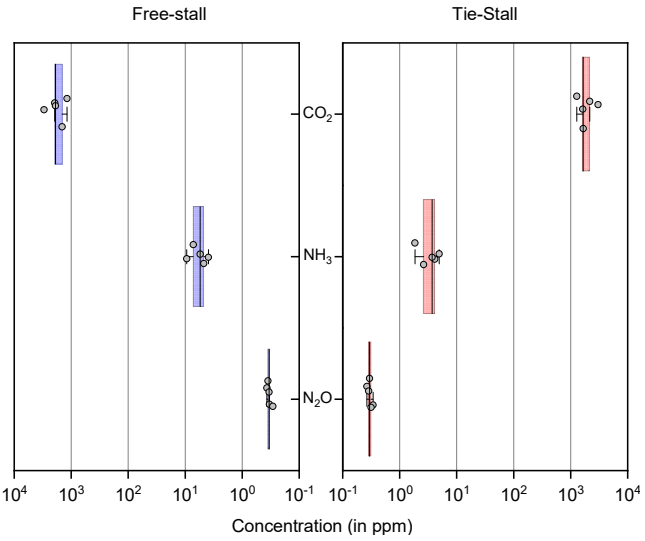

Figure 1. Concentrations of gas measured in free-stall (blue, n=5) and tie-stall (red, n=5) dairy barns. Each point represents a barn, and the middle bar represents the median.

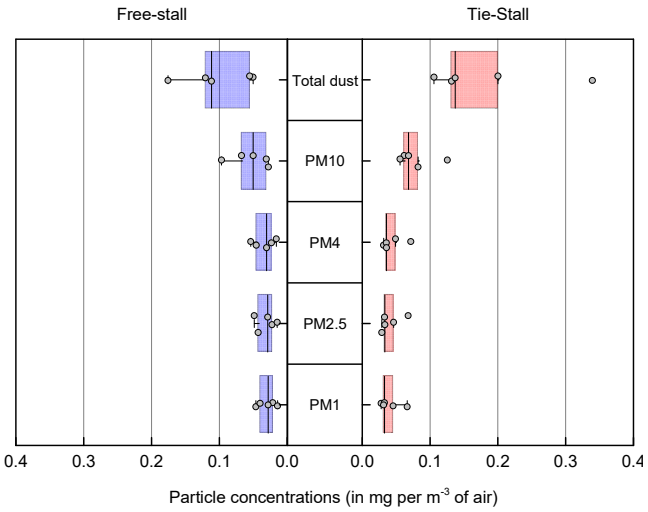

Figure 2. Airborne concentrations of dust particles measured in free-stall (blue, n=5) and tie-stall (red, n=5) dairy barns.

concentration values ranged from 0.035 to 0.160 mg m^{-3} for tie stalls and from 0.016 to 0.129 mg m^{-3} for free stalls. For total airborne dust concentrations, the highest concentrations were up to 0.417 mg.m^{-3} in tie-stall barns. The highest value for total dust in free-stall barns was 0.238 mg.m^{-3}. No statistical differences were detected.

Concentrations of Bioaerosols

Total bacteria concentrations ranged between 8.47×10^6 and 6.69×10^8 copies m^{-3} for tie-stall barns and between 5.33×10^6 and 2.12×10^8 copies m^{-3} for free-stall barns (fig. 3). For *Penicillium/Aspergillus*, concentrations were from 7.03×10^5 to 2.61×10^8 copies m^{-3} for tie stalls and from 2.86×10^5 to 4.64×10^8 copies m^{-3} for free stalls. Archaea concentration values were between 1.55×10^6 and 4.62×10^7 copies m^{-3} for tie-stall barns and between 1.96×10^6 and 2.39×10^7 copies m^{-3} for free-stall barns. No statistically significant difference was detected for bioaerosols.

Specifically targeted fecal indicators and etiological agents were also present in bioaerosols, except for *Listeria monocytogenes*. However, some of these targeted microorganisms were not detected by qPCR in some samples, which could not be included in figure 3. *E. coli, Enterococcus* sp., and *C. perfringens* were detected in high concentrations of bioaerosols. Airborne *E. coli* concentrations varied between 493 and 4.36×10^4 copies m^{-3} while *Enterococcus* spp. Showed concentrations between 5,040 and 2.05×10^6 copies m^{-3}. As for *C. perfringens*, concentration values were between 400 and 4.70×10^4 copies m^{-3}. SR was detected at concentrations ranging from 1.21×10^5 to 7.70×10^8 copies m^{-3}. For *A. fumigatus*, values ranged from 1.82×10^4 to 4.82×10^8 copies m^{-3}. *S. aureus* was detected at concentrations ranging from 7.95×10^4 to 1.39×10^7 copies m^{-3}, while *Coxiella burnetii* (one positive barn for tie stalls, four positive barns for free stalls) concentration values were between 2.11×10^4 and 6.80×10^7 copies m^{-3}. Finally, for airborne *Klebsiella pneumoniae* concentrations ranged from 5,300 to 2.47×10^5 copies m^{-3}. No significant statistical difference was found for fecal indicators or etiological agents.

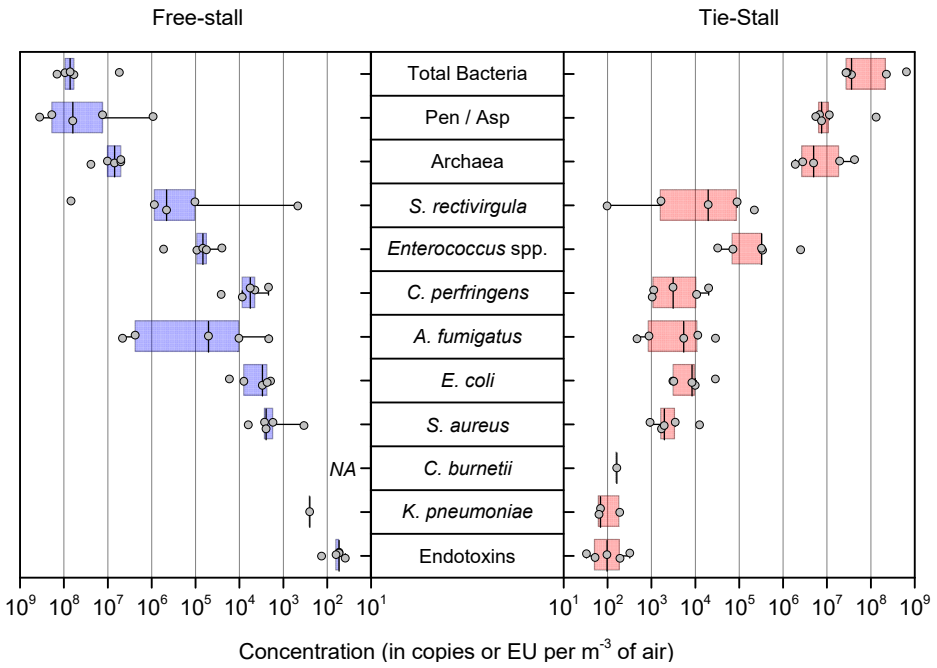

Figure 3. Airborne concentrations of the different indicators of air quality, fecal indicators, as well as etiological agents evaluated in free-stall (blue, n=5) and tie-stall (red, n=5) dairy barns.

Figure 3 also shows results for endotoxin concentrations in the air of the visited dairy barns. Results for tie stalls are more variable than for free stalls, with values between 21 and 392 EU m^{-3}. Free-stall barn concentrations include values ranging between 12 and 177 EU m^{-3}. No statistically significant difference was found for endotoxin.

Concentration of Biological Agents in Soiled Bedding Material

Total bacteria median concentrations did not differentiate between the two types of housing, with values ranging from 3.18×10^{11} to 1.87×10^{12} copies g^{-1} for tie stalls and between 6.36×10^{10} and 4.54×10^{12} copies g^{-1} for free stalls (fig. 4). For *Penicillium/Aspergillus*, median concentrations in free-stall barns were significantly higher ($p = 0.023$, Kruskal-

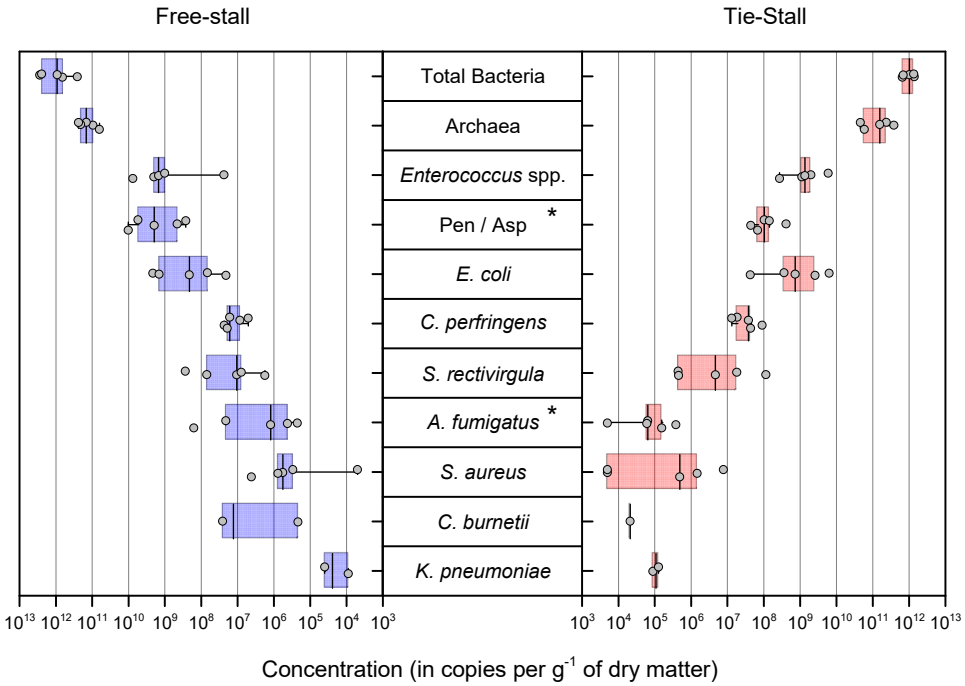

Figure 4. Concentrations of the different indicators and etiological agents found in the soiled bedding material of free-stall (blue, n=5) and tie-stall (red, n=5) dairy barns. Statistical differences between free-stall and tie-stall barns are highlighted by an asterisk ($p < 0.05$, Kruskal-Wallis test).

Wallis test), with concentrations ranging from 1.08×10^8 to 2.62×10^{10} copies g^{-1} compared to values from 5.38×10^6 to 6.52×10^8 copies g^{-1} in tie-stall barns. Archaea median did not differentiate between the two types of housing, with values ranging from 2.66×10^{10} to 4.48×10^{11} copies g^{-1} for tie stalls and between 4.48×10^{10} and 3.67×10^{11} copies g^{-1} for free stalls. For Pen/Asp ($p = 0.023$, Kruskal-Wallis test), concentrations in soiled bedding were significantly higher in free-stall barns.

Most of the fecal indicators and specific etiological agents previously listed (table S1) were detected by qPCR, except for *Listeria monocytogenes*. Results for soiled bedding materials are shown in figure 4. Some of these targets were not as concentrated as the others; some samples/replicates were below the limit of detection and are not illustrated in the figure 4. *E. coli, Enterococcus* spp., and *C. perfringens* were detected in high concentrations in soiled bedding material. *E. coli* concentrations varied between 7.96×10^5 and 1.88×10^{10} copies g^{-1}, while *Enterococcus* spp. concentrations were between 2.05×10^6 to 1.03×10^{10} copies g^{-1}. As for *C. perfringens*, concentration values ranged between 1.88×10^6 and 9.36×10^7 copies g^{-1}. *Saccharopolyspora rectivirgula (SR), Aspergillus fumigatus, Staphylococcus aureus, Coxiella burnetii,* and *Klebsiella pneumoniae* were also detected in samples of soiled bedding material. SR was detected at concentrations ranging from 1.21×10^5 to 7.70×10^8 copies g^{-1} while for *A. fumigatus*, values ranged from 1.82×10^4 to 4.82×10^8 copies g^{-1}. *S. aureus* was detected at concentrations ranging from 7.95×10^4 to 1.39×10^7 copies g^{-1}. As for *Coxiella burnetii*, concentrations were between 2.11×10^4 and 6.80×10^7 copies g^{-1}. Finally, for *Klebsiella pneumoniae*, concentrations in soiled bedding material ranged from 5,300 to 2.47×10^5 copies g^{-1}. For *A. fumigatus*, ($p = 0.043$, Kruskal-Wallis test), concentrations in soiled bedding were significantly higher in free-stall barns. Otherwise, no significant statistical difference was found.

Association between Environmental Factors and Bioaerosols

An NMDS model is illustrated in figure 5 (stress = 0.037). The *permanova* analysis shows no significant difference between the tie-stall barns (red) and the free-stall barns (blue). Tie-stall housing seems, however, to be associated with higher concentrations of total airborne bacteria, archaea, *Enterococcus* spp., and endotoxins. Additionally, the correlogram (fig. 6) shows strong and significant correlations between concentrations of these air quality indicators. On the other hand, free-stall barns seem to have higher concentrations of airborne *Penicillium /Aspergillus*, *A. fumigatus,* and *Saccharopolyspora rectivirgula.* Again, the correlogram indicates strong and significant correlations between concentrations of Pen/Asp, *A. fumigatus,* and SR. Other air quality indicators (total dust, *E. coli*, *C. perfringens,* gases, and *S. aureus*) were not exclusive to any of the two types of

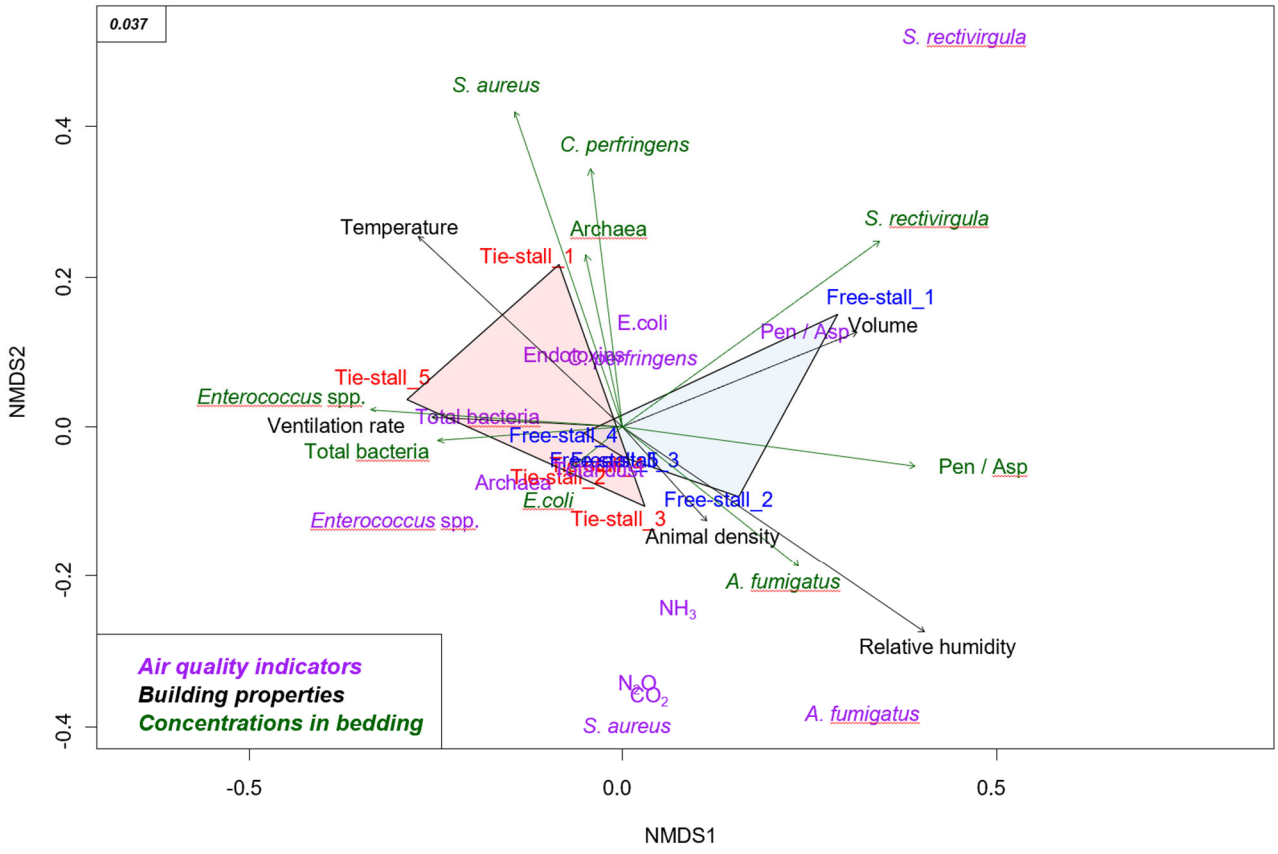

Figure 5. Non-metric multidimensional scaling (NMDS) performed on the concentrations of the different indicators of air quality measured in the present study (purple) and calculated using the Gower distance. Free-stall and tie-stall dairy barns are shown in blue and red, respectively with the limits of both groups being represented by the associated convex hulls. Barn properties and concentrations of indicators and etiological agents from the soiled bedding material were fitted to the NMDS model and appears in black and dark green, respectively.

housing. However, for *E. coli* and *C. perfringens,* strong and significant correlations were found with endotoxins (fig. 6). Tie-stall air quality indicators seem to be mainly influenced by temperature and ventilation rate, whereas indicators in free-stall housing seem to be influenced by relative humidity, volume, and animal density (fig. 5). Concentrations of SR and *A. fumigatus* are highly correlated to their concentrations in the bedding (fig. 6). The NMDS model also highlights some outliers. Two visited tie-stall barns (Tie-stall 1 and Tie-stall 5) stand out in terms of some specific air quality indicators. Tie-stall 1 showed high concentrations of *E. coli,* endotoxins, and *C. perfringens* in the air and *C. perfringens* in the soiled bedding material. *S. aureus* and archaea were also particularly present in higher concentrations in the bedding of this barn. Tie-stall 5 was associated with high concentrations of airborne *Enterococcus* and total bacteria, which seem to be linked to the ventilation rate. *Enterococcus* and total bacteria in the bedding also seem to be linked with these results, but no significant correlation was found. Two outliers were found in free-stall dairy barns (Free-stall 1 and Free-stall 2), with high concentrations of Pen/Asp and SR in bioaerosols and bedding material for free-stall 1 and high concentrations of *A. fumigatus* in bioaerosol and bedding material for free-stall 2. Even though *A. fumigatus* in the air and the bedding material seems to be highly influenced by relative humidity in the model, no significant correlation was found.

Discussion

The main objective of the present study was to analyze and describe air quality in dairy farms using tie-stall housing and those using free-stall housing. Several air quality indicators have been measured, such as concentrations of dust particles (PM1, PM2.5, PM4, PM10, total dust), total

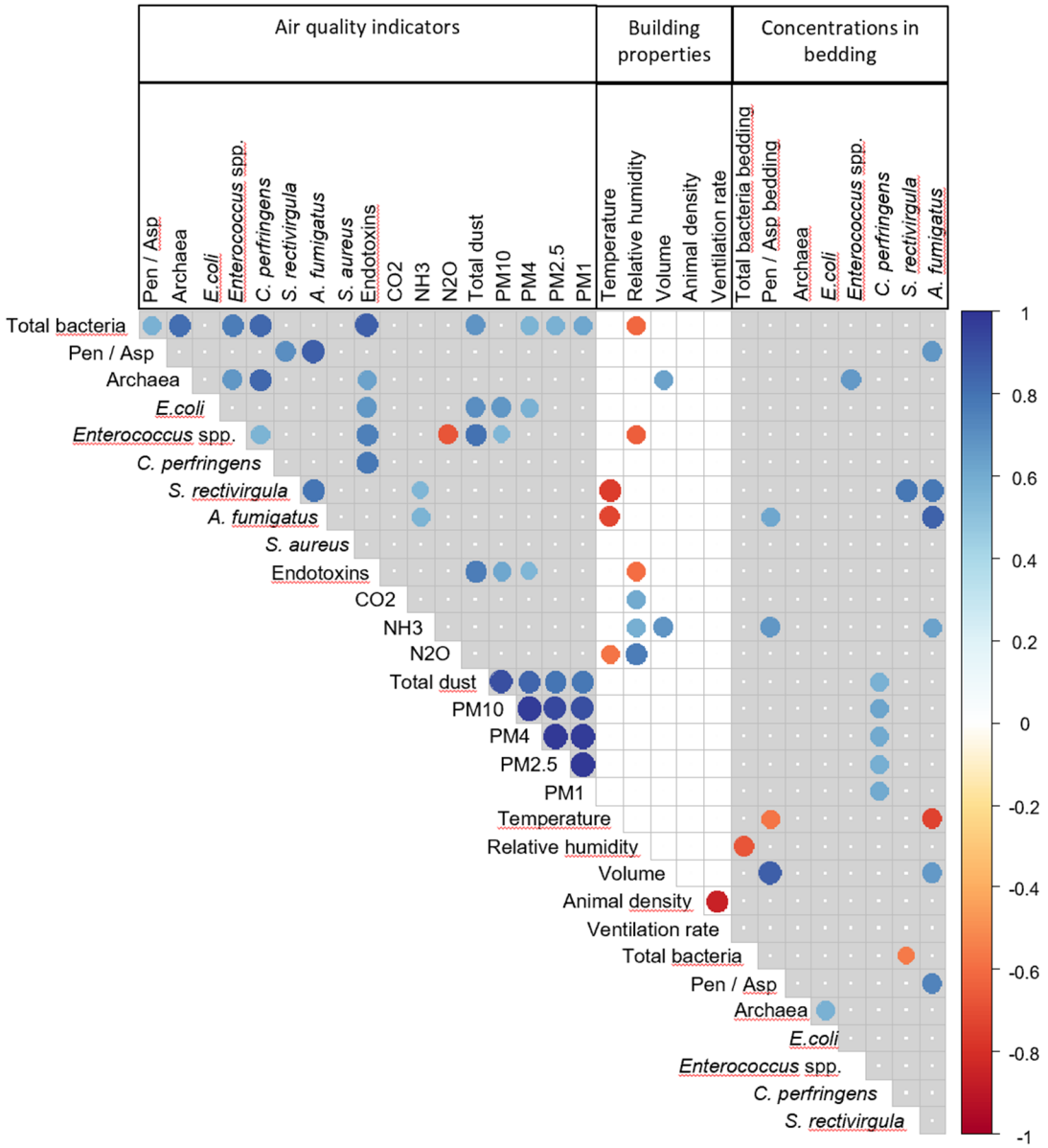

Figure 6. Correlogram of the concentrations of the different indicators of air quality, the barn properties and the concentrations observed in bedding. Two by two correlations were estimated using the Spearman correlation coefficient, and only those whose σ > 0.55 are displayed (= P < 0.10).

bacteria, *Penicillium/Aspergillus*, archaea and endotoxins. Some etiological agents were detected and quantified to further characterize the bioaerosols of dairy production. Finally, the impact of the different environmental factors on bioaerosols was assessed.

In the present study, concentrations of dust and bioaerosols were measured in the absence of workers' activities for both types of housing. Total dust concentrations were low compared to the ones already reported in the literature, with values between 0.2 and 9.5 mg m^{-3} (Louhelainen et al., 1987; Virtanen et al., 1988; Basinas et al., 2015). Although concentrations were not statistically different between both types of dairy barns, one tie-stall barn stands out in terms of concentrations for all particle size fractions. The concentrations observed in the 10 visited dairy barns were far from the threshold limit value (TLV) of OSHA (10 mg/m^3). Low dust concentrations of the present study could be explained by the absence of workers' activities at the time of the sampling. Concentrations would also necessarily increase during spreading activities (bedding material or feed). The present study, however, focuses on the characterization of air quality at baseline values, so dust generated from activities would add variables to the model (e.g., the type of machinery used for spreading bedding material impacts the generation of dust). Endotoxin concentrations in previous studies reported values between 21 and 34,800 EU m^{-3} in dairy barns (Kullman et al., 1998; Samadi et al., 2012; Basinas et al., 2015). The values found here are low compared to these studies, but some visited dairy barns still exceeded the exposure limit value of 90 EU m^{-3} recommended in the Netherlands (Health Council of the Netherlands, 2010). The use of face masks is advisable but not adapted to these kinds of environments. Thus, ventilation management and dust mitigation techniques must be considered.

The values obtained for total bacteria are consistent with previous studies, with values up to 10^8 copies m^{-3} (Blais Lecours et al., 2012). As for Pen/Asp and archaea, concentrations were higher than previous published data, with maximum values of 10^6 copies m^{-3} for Pen/Asp (Mbareche et al., 2019) and 10^6 archaeal 16S rRNA genes m^{-3} (Blais Lecours et al., 2012). Some characteristics of the visited barns (straw litter, cold season, mechanical ventilation) as well as environmental conditions (temperature and relative humidity) may have impacted Pen/Asp and archaea concentrations. Dairy barns are known to be humid environments, and a high relative humidity in bedding has been linked with the development of molds like *Aspergillus fumigatus*. (Gregory et al., 1963; Whitlow and Hagler, 2008), which would explain higher airborne mold concentrations compared to other livestock production such as poultry and swine operations. Reduced ventilation during the winter may also impact these findings. The high amounts of methanogenic archaea present in the cow's gut explain the important presence of archaea in the air. Exposure to these microorganisms can lead to a pulmonary immunogenic response, as shown in a murine model (Blais Lecours et al., 2011).

General air quality indicators (total dust, PM10, PM4, PM2.5, PM1, total bacteria, Pen/Asp, archaea, endotoxins) as well as gas (CO_2, NH_3, and N_2O) concentrations showed no significant difference between both types of dairy barns. The statistical difference in animal density (table 1) may explain why no major differences in air quality were detected, since free-stall barns are generally larger and dust particles and bioaerosols generated from cow movement could be diluted in a larger volume of air. However, if cow movements in free-stall barns were responsible for production of dust and bioaerosols, the gain made by the dilution effect in terms of air quality might have been nullified. Nevertheless, tie-stall barns showed more variation (86.1%) than free-stall barns (79.1%) (table S2). For comparison of dairy barns with different dimensions, with a perspective of the environmental impact instead of occupational health, it could be appropriate to calculate individual cow emissions of dust and bioaerosols, by considering animal density as well as ventilation rates. These emissions of dust and bioaerosols are expressed per hour per cow (e.g., copies h^{-1} cow^{-1}). For the present study, the emission data showed no significant difference between both housing types (table S3). Higher emissions in tie-stall dairy barns could be explained by the fact that cows are constantly stepping on bedding material. Though the smaller size of the barns would mean a better chance of achieving a better air change rate. Generally, the effect of cow movement on air quality might be mitigated because of animal density, and the absence of a difference in emissions might be due to the air change rate.

Several fecal indicators, as well as etiological agents, were detected and quantified in the air and the soiled bedding material. The presence of *C. burnetii* was revealed in four dairy barns, specifically in one bioaerosol and three soiled bedding material samples. The bacterium *C. burnetii* is linked with cases of Q fever in dairy barn workers and abortions in ruminants, with several cases reported in Quebec (Turcotte et al., 2021). The transmission of *C. burnetii* in dairy barns is not fully understood, but the main source appears to be amniotic fluids (Woldehiwet, 2004; Eldin et al., 2017). The present study is the first to report the presence of *C. burnetii* and *C. perfringens* in the air of dairy barns. Considering the high amount of fecal matter in dairy barns, fecal indicators were expected in the air. Hence, *E. coli*, *Enterococcus* sp., and *C. perfringens* were detected in high concentrations in bioaerosols. *Saccharopolyspora rectivirgula* was found in all visited farms and in large quantities both in the air and the soiled bedding material. Past studies reported concentrations of *S. rectivirgula* up to 10^7 copies m^{-3} of air (Duchaine et al., 1999; Blais Lecours et al., 2012), which is consistent with the findings of the present study. This actinomycete is mostly found in damp straw and can cause farmers' lung in exposed workers (Pepys et al., 1990). *A. fumigatus,* a mold responsible for aspergillosis (CDC, 2022), was also found in all ten visited barns. With the high concentrations of *A. fumigatus* (up to 10^5 copies m^{-3} of air) revealed by Mbareche et al. (2019), it was expected to find similar concentrations. However, similarly to Pen/Asp, concentrations of the present study were much higher, which can be caused by environmental condition at the time of the samplings and selection criteria for dairy barns. *S. aureus* and *K. pneumoniae* were also found in a few farms, both in the air and soiled bedding material. Both bacteria are associated with opportunistic infections in humans (Lin and Peterson, 2010; Paczosa and Mecsas, 2016). Certain strains can cause

udder inflammation and clinical mastitis. For example, specific *spa* types (staphylococcal protein A) of S. *aureus* are linked with reoccurring clinical mastitis in Quebec (Veh et al., 2015; Pichette-Jolette et al., 2019; Demontier et al., 2021). *S. aureus* culture was done from bioaerosol and soiled bedding material samples to detect the presence of these *spa* types in the visited farms. *Spa* typing was then done to compare the protein A sequences to those most commonly present in clinical mastitis cases. No *spa* types linked with mastitis were found in the isolates of the present study (data not shown). These results shed new data about the biological contents of airborne particles.

The NMDS model shows that the two groups (tie-stall and free-stall) overlap, and that air quality is not significantly different between the two types of barns. However, the model shoes that Pen/Asp and *A. fumigatus* concentrations seemed to be linked with relative humidity, which has been shown to have an effect on mold proliferation (Gregory et al., 1963; Whitlow and Hagler, 2008). Additionally, relative humidity tended more toward the free-stall group, which could explain the higher concentrations in the bedding for Pen/Asp ($p=0.023$) and *A. fumigatus (*$p=0.043$). Only airborne SR and *A. fumigatus* concentrations seemed to be influenced by the concentrations in soiled bedding material (fig. 5), which is supported by the correlogram (fig. 6). The NMDS model also shows how ventilation rates could explain air quality in tie-stall buildings (smaller buildings and air volume), whereas the air quality of free-stall buildings could be explained by animal density. There was a significantly lower animal density in free-stall buildings ($p < 0.009$). Some outliers were also discovered, but with the high number of variables, this is to be expected.

The small study population implies that the statistical model can be improved with additional samples. However, this study provides a proof of concept for the comparison of the air quality between the two types of barns. Adding more barns to these results would also improve the representativeness of the participating herds in the many barns in the region of Quebec.

CONCLUSIONS

The present study aimed at describing air quality in free-stall and tie-stall dairy farms and evaluating whether the increased movement of cows results in higher dust and bioaerosol concentrations. The concentrations of airborne dust, total bacteria, *Penicillium/Aspergillus,* archaea, endotoxins, and gases did not differentiate the two types of housing. The comparison between the two types of farms remains interesting as a proof of concept and to detect any trends. Indicators of fecal contamination (*E. coli*, *Enterococcus* sp., *C. perfringens*) as well as several etiological agents (*A. fumigatus*, *S. rectivirgula*, *S. aureus*, *C. burnetii*, and *K. pneumonia*) were detected in the air of dairy barns. These results add new insights on the biological components of bioaerosols in dairy farms. Concentrations in the air seemed to be associated with the amounts in soiled bedding material for several indicators and etiological agents evaluated in the present study. Ventilation rate and animal density also seemed to have an important effect on the air quality of tie-stall barns and free-stall barns, respectively. This study brings new results about air quality and the characterization of bioaerosols in dairy barns. While the sampling size limits statistical power for comparisons, this study offers preliminary results about potential differences in air quality between tie-stall and free-stall barns. Future studies would help understand the impacts of those findings with regards to human and animal health outcomes. Individual exposure to certain air contaminants would help assess the response to poor air quality among workers. This would help determine new exposure limit values, since few of them are available for airborne biological particles. Replicating this study in controlled environments (for each type of dairy barn) with repeated measurements would be a way to reduce the number of variables.

SUPPLEMENTAL MATERIAL

The supplemental materials mentioned in this article are available for download from the ASABE Figshare repository at: https://doi.org/10.13031/25009535

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