Characterization of Bioaerosols from Dairy Barns: Reconstructing the Puzzle of Occupational Respiratory Diseases by Using Molecular Approaches

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To understand the etiology of exposure-related diseases and to establish standards for reducing the risks associated with working in contaminated environments, the exact nature of the bioaerosol components must be defined. Molecular biology tools were used to evaluate airborne bacterial and, for the first time, archaeal content of dairy barns. Three air samplers were tested in each of the 13 barns sampled. Up to 10⁸ archaeal and 10⁹ bacterial 16S rRNA genes per m³ of air were detected. Archaeal methanogens, mainly *Methanobrevibacter* species, were represented. *Saccharopolyspora rectivirgula*, the causative agent of farmer’s lung, was quantified to up to 10⁷ 16S rRNA genes per m³ of air. In addition, a wide variety of bacterial agents were present in our air samples within the high airborne bioaerosol concentration range. Despite recommendations regarding hay preservation and baling conditions, farmers still develop an *S. rectivirgula*-specific humoral immune response, suggesting intense and continuous exposure. Our results demonstrate the complexity of bioaerosol components in dairy barns which could play a role in occupational respiratory diseases.

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   icrobial flora from natural sources, such as water, soil, plants, and animals, are well known and studied (14, 19, 41, 43). Yet little information on bioaerosols is available. This lack of knowledge impedes the understanding of the bioaerosol-related respiratory diseases etiology (3, 18, 46). Indeed, the airway mucosa is a primary entry site for toxic and pathogenic factors, but what the workers are exposed to and how it impacts respiratory health are poorly addressed. From what is currently known, the presence of microbial components assessed with simple culture methods cannot explain the whole variety of respiratory diseases. Nonviable agents of bioaerosols, such as toxins and antigens, can induce sensitization or toxic diseases (3, 22). Moreover, due to a lack of standardization between air samplers used for exposure assessment purposes, the robustness of the sampling protocol is worrisome.

Complex environments, such as agricultural facilities, are used to study the causal effects of bioaerosol agents on respiratory health, since several sources of biological material potentially associated with lung diseases are present. As an example, our team discovered high concentrations of archaea in swine barn bioaerosols (33), revealing for the first time a human exposure to archaea through the airborne route. Those archaea (8) and their subcomponents (36) were revealed to have a significant immunogenic potential. Molecular biology is the most efficient approach allowing the evaluation of exposure to archaea and any other noncultivable microorganisms that can have an immunogenic effect on human health (6, 11, 26, 33). Indeed, molecular methods were used in several studies to characterize airborne bioaerosols from various agricultural environments (20, 35).

The main objective of this project was to characterize the bacterial and archaeal loads from the bioaerosols of dairy barns, with special emphasis on *Saccharopolyspora rectivirgula* (a farmer’s lung agent) (13) and archaeal species known to have an immunogenic potential (8), using molecular approaches. Secondary objectives included a comparison between different types of air samplers, particle size-selective analysis of bioaerosols, and workers’ IgG responses to *S. rectivirgula* as a marker of exposure.

MATERIALS AND METHODS

Sampling, collecting, and processing methods. Airborne dust was sampled at one site each from 13 Holstein dairy barns (eastern Quebec, Canada). Three different air samplers were used, namely, the Institute of Occupational Medicine cassettes (SKC, Ancaster, ON, Canada) or IOM samplers, loaded with 25-mm-diameter gelatin membranes (SKC) and plugged into a Giliar-5 pump (Levitt-Sécurité Limitée, Dorval, QC, Canada), at 2 liters/min (50% cutoff size of 4.0 μm); the Coriolis (Bertin Technologies, Montigny-le-Bretonneux, France), which collects 100% of the particles of 4.4 μm, at 100 liters/min and loaded with 15 ml of 0.9% saline solution; and the NIOSH two-stage bioaerosol cyclone (BC 251) sampler (31, 42), plugged into an AirCon-2 pump (Gilian) and sampling at 10 liters/min for particulate size separation. The size distribution for each stage (50% cutoff size) of the NIOSH sampler was as follows: 2.1 μm for the first stage and 0.41 μm for the second stage. The third stage was composed of a 0.4-μm polycarbonate filter. Air samples from dairy barns were collected in dairy barns next to the cows during the winter season, 1 m above the floor and as far as possible from ventilation sources, for 4 to 5 h during morning or evening cow milking. One sampling per dairy barn was conducted.

Samples from IOM cassettes were treated as previously described (33). Liquid samples from the Coriolis samplers were divided into aliquots of 1.5 ml and were centrifuged (10 min, 21,000 × g, room temperature). Filters from the NIOSH samplers were transferred in sterile tubes in which...
TABLE 1 Primers, probe, and GC clamps used in the study

<table>
<thead>
<tr>
<th>Primer, probe, or GC clamp</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Reference</th>
</tr>
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<td>CCG AGG GTG AGR GRY GAA</td>
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</tr>
<tr>
<td>A976R</td>
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<tr>
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<tr>
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<td>GAC ARC CAT GCA SCA CTT G</td>
<td>4</td>
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<tr>
<td>Probe EUB</td>
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<td>4</td>
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<tr>
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<td>40</td>
</tr>
<tr>
<td>Sac-183R</td>
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<td>40</td>
</tr>
<tr>
<td>A333F</td>
<td>TTC AGG CCC TAC GGG</td>
<td>38</td>
</tr>
<tr>
<td>A751R(GC)</td>
<td>GGG CCG GGC GGC CCC CCC GGG CCC CCC CCC CCC CCC CCC</td>
<td>5</td>
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</table>
| GC clamp archaea          | GGG CCG GCG GGC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC 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Antigen-specific immunoglobulin G detection. As previously described, total IgGs specific for *Saccharopolyspora rectivirgula* from plasma of dairy barn workers and control subjects were measured by indirect enzyme-linked immunosorbent assay (ELISA) (10). Plasma samples from workers and control subjects were diluted 1/500 before they were added to the antigen (crude extract)-coated plate, prepared as previously described (15). Blank, negative, and positive controls were also added to each plate. The enzyme reaction was stopped when the positive control for each plate reached the same optical density (OD). Plasma samples were considered positive (1+) for the antigen when the optical density was higher than the OD 95% confidence interval of the control samples. The reaction intensity from control subjects and workers was scored from − (no reactivity) to 4+ (strong reactivity) depending on the OD obtained. A score of 2+ corresponded to an OD from samples that doubled the control sample average, whereas 3+ or 4+ ODs tripled (3+) or quadrupled (4+) it. Samples were classified as negative (−), total positive (1+ to 4+), or strongly positive (3+ and 4+). Although the plasma IgG should be specific to the *S. rectivirgula* antigen, cross-reactions between epitopes from different antigens could not be prevented in this technique.

Statistical analysis. Quantitative data were expressed using the average and the standard error of the mean (SEM). Total archaeal and bacterial data from the three different samplers were analyzed using a mixed analysis of variance (ANOVA) with two experimental factors, one associated with the comparison between samplers (factor fixed) and one associated with the amount of this bacterium in each dairy barn sampled. Indeed, averages of 9.2 \times 10^9 archaeal 16S rRNA genes per m^3 of air were found in the 13 dairy barns sampled (IOM sampler) (Fig. 1). Archaeal detection ranged from 8.6 \times 10^9 to 3.5 \times 10^9 16S rRNA genes per m^3 of air, while the amount of bacteria ranged from 1.81 \times 10^10 to 1.05 \times 10^11 16S rRNA genes per m^3 of air (IOM sampler). In addition to broad-spectrum quantification of bacterial DNA, we also performed agent-specific detection of *S. rectivirgula* since bioaerosols from dairy barns have historically been shown to contain this agent, a cause of the farmer’s lung disease. Detection of *S. rectivirgula* by quantitative PCR showed high differences between the amounts of this bacterium in each dairy barn sampled. Indeed, an average of 1.4 \times 10^8 16S rRNA genes of *S. rectivirgula* per m^3 of air was found, but this level ranged from below the limit of detection (LOD) to 1.3 \times 10^7 gene copy numbers per m^3 of air (Fig. 2).

Bioaerosol size distribution studied with the NIOSH sampler showed that the majority of microorganisms sampled were found in the first stage, i.e., particles with an aerodynamic diameter of over 2.1 \mu m (Fig. 3). Indeed, averages of 9.2 \times 10^5, 1.7 \times 10^4, and 9.0 \times 10^3 archaeal 16S rRNA genes per m^3 of air were found in stages 1 to 3, respectively. The same trend was observed for bacteria, with averages of 3.5 \times 10^7, 3.4 \times 10^6, and 7.7 \times 10^5 16S rRNA genes per m^3 of air found in stages 1 to 3, respectively. There was no correlation between the quantity of airborne archaea and that of airborne bacteria in dairy barns, with a correlation coefficient (r) of 0.2629 for the IOM sampler, 0.0839 for the NIOSH sampler, and 0.0464 for the Coriolis sampler (Fig. 4).
Methanogens and various species of bacteria aerosolized in dairy barns. Airborne archaeal and bacterial species detected in dairy barns were identified using PCR-DGGE on 16S rRNA genes from both domains. Since similar archaeal and bacterial biodiversity profiles were observed on DGGE for every sampler (data not shown), we used only total DNA from IOM cassettes for DGGE analysis. For archaeal analysis (Table 2), 17 different band classes were observed among DGGE profiles from the 13 dairy barns sampled. Clustering within archaeal DGGE profiles from each barn showed high homology (80% similarity and higher) for 7 barns out of 13, and the percentage similarity was lower than 40% for 2 barns out of 13. As suspected, the five extracted and sequenced bands had high identity homology (95 to 98%) with the Methanobacteriaceae group. The genus Methanobrevibacter represented 100% of all DGGE bands sequenced. M. smithii, detected in 2 barns out of 13, and Methanobrevibacter ruminantium, detected in every barn sampled, were the archaeal species identified by sequencing (Table 2).

The characterization of airborne bacterial species from dairy barns (Table 2) revealed a DGGE profile of 22 different band classes, from which 8 were extracted and sequenced. All dairy barns’ bacterial DGGE profiles clustered together with 65 to 92% similarity. Two of the sequenced bands (1 and 4) included DNA from two different bacterial species. DGGE bands had identity homology (94 to 99%) with various bacterial species, namely, Staphylococcus gallinarum, Clostridium ileocola, Oxalobacter sp., Agrobacterium tumefaciens, Clostridium quinii, Staphylococcus sp., Agrobacterium sp., Corynebacterium variabile, and Corynebacterium xerosis. All of the bacterial DGGE-sequenced bands except for band 5 were found in 12 or 13 barns out of 13 (Table 2).

Dairy barn workers were sufficiently exposed to airborne S. rectivirgula to induce a humoral response. S. rectivirgula is a major airborne microorganism found in dairy barns, and, consequently, the workers’ airways can be exposed to this microorganism. We actually found up to $10^7$ 16S rRNA genes of S. rectivirgula per m$^3$ of air in this work environment. Thus, we determined if plasma of workers from the 13 dairy barns visited contained IgG specific to this antigen, a method often used by our team to confirm exposure (10, 12, 32). A total of 85.7% of plasma samples from control subjects had a negative IgG response to S. rectivirgula, compared to 62.1% for samples from dairy barn workers. Total positive samples reached 14.3% for control subjects and 37.9% for workers. From these positive samples, only 2.9% were considered strongly positive for control subjects, compared to 10.3% for the workers. Overall, a higher number of workers than control subjects had positive plasma S. rectivirgula-specific IgGs (Table 3).

**DISCUSSION**

Unlike most natural microbial habitats, such as water and soil, bioaerosols have not been well studied and characterized. Since hay and straw are important sources of microorganisms in dairy barns, most studies have focused on fungus, endotoxin, and actinomyces airborne contamination (16, 28, 30). However, as reported earlier in swine barns (33, 34), using molecular biology tools, we demonstrated that bioaerosol biodiversity is much more complex than expected. In the current study, we detected airborne archaeal species, which can be immunogenic agents (8), for the first time in dairy barns. Moreover, we characterized the airborne bacterial diversity of dairy barns, focusing on the occupational respiratory diseases. Results show strong similarities between data from different air samplers. It was also revealed that, despite modern hay baling and management practices, dairy barn workers are exposed to high concentrations of S. rectivirgula, the causative
agent for farmer’s lung, and a significant proportion of workers develop humoral responses against that bioaerosol agent. These results provide the first culture-independent data on dairy barn bioaerosol composition. They highlight the lack of knowledge on bioaerosol agents in working environments and the importance of a better biodiversity assessment.

Results from bioaerosol studies that were obtained with different air samplers are comparable. Indeed, quantitative data from the IOM, the NIOSH, and the Coriolis samplers were similar (Fig. 1). Qualitative data from DGGE profiles of different samplers also showed similar data (data not shown). These results are not surprising considering the fact that the majority of the particles captured had an aerodynamic diameter of 2.1 μm (Fig. 3), which is in the capture range of all three samplers (1, 7, 31). Therefore, even while using different particulate trapping systems, data were similar, so results from different studies can be compared. No matter which air sampler is used on the field, analysis should lead to similar results.

Bioaerosols of dairy barns contain high concentrations of various species of bacteria and archaea. According to King et al., *Methanobrevibacter* (RO and SGMT clades) and *Methanosphaera* are the two main archaeal genera found in the rumens of Holstein cows. Two different species of *Methanobrevibacter* were detected by PCR-DGGE, including *Methanobrevibacter smithii*, an immunogenic species of archaea (8). Even if *Methanosphaera stadtmanae* is one of the main species found in cows’ rumens, it has not been detected in the air of the dairy barns sampled in this study. However, according to Yu et al., PCR-DGGE archaeal biodiversity results depend on the primer set used (44). The main bacterial genera in the rumens of Holstein cows are the *Cytotogalla-Flavobacterium-Bacteroides* phylum, *Prevotella*, *Ruminobacter*, and *Clostridium* (43). The majority of our bacterial airborne results did not include these species, which are probably due to bacterial sources other than cow manure. Indeed, a recent study on bioaerosols’ emission from an open-freestall dairy barn operation (17) showed a wide bacterial diversity. As suspected, airborne archaeal and bacterial biodiversity showed high similarity within dairy barns sampled, although clustering percentages were higher for the bacterial analysis. This can be explained by the higher number of archaeal bands that appeared sporadically on the gel compared to that of bacteria. Interestingly, we found no correlation between bacterial and archaeal bioaerosols in dairy barns (Fig. 4), while this correlation was found to be positive in swine barn facilities (33). This can be explained by the consistency of airborne archaeal concentrations between the different dairy barns sampled (Fig. 4). According to Jeyanathan et al., the archaeal community in cows’ rumens is constant, less variable, and less diverse than the bacterial community (26). Indeed, the archaeal source, the cows’ rumens, is the most constant source of bioaerosols in a dairy barn. Bacterial sources in dairy barns are probably more diversified (hay, straw, water, manure, grain) than those in swine barns, justifying the lack of correlation between archaeal and bacterial quantities in dairy barns. These results increase the knowledge on dairy barns’ biological burden. What are the potential impacts of these newly found airborne microorganisms on the respiratory health of workers? Can they play a synergic role with other bioaerosol components in lung diseases?

Dairy barn workers are still exposed to *S. rectivirgula*, the causal agent of farmer’s lung. Even though recommendations are frequently made to farmers about hay preservation to minimize the risk of contamination by the actinomycete (9, 21, 23, 39), *S. rectivirgula* is still present in high concentrations in the air in dairy barns (Fig. 2). Indeed, up to 107 CFU per m³ were found by culture in Quebec dairy barns in 1999 (16) and up to 1 million 16S *S. rectivirgula* rRNA genes per m³ were found in this study using molecular biology. Bioaerosol contamination by a high concentration of *S. rectivirgula* cells has led to signifi-

### TABLE 2 Sequence matches for bands from DGGE gels containing dairy barn archaeal and bacterial DNA

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Band no.</th>
<th>Frequency (no. of positive samples)</th>
<th>Most similar sequence</th>
<th>bp</th>
<th>% similarity</th>
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<tr>
<td>Archaeal</td>
<td>1</td>
<td>5</td>
<td><em>Methanobrevibacter</em> sp. JQ267743</td>
<td>302</td>
<td>97</td>
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<td></td>
<td>2</td>
<td>2</td>
<td><em>Methanobrevibacter smithii</em> JQ267744</td>
<td>313</td>
<td>97</td>
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<tr>
<td></td>
<td>3</td>
<td>9</td>
<td><em>Methanobrevibacter</em> sp. JQ267745</td>
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<td>95</td>
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<tr>
<td></td>
<td>4</td>
<td>12</td>
<td><em>Methanobrevibacter ruminantium</em> JQ267746</td>
<td>305</td>
<td>95</td>
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<td></td>
<td>5</td>
<td>13</td>
<td><em>Methanobrevibacter ruminantium</em> JQ267747</td>
<td>316</td>
<td>98</td>
</tr>
<tr>
<td>Bacterial</td>
<td>1</td>
<td>13</td>
<td><em>Staphylococcus gallinarum</em> JQ267748</td>
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<td>99</td>
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<tr>
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<td>13</td>
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<td>99</td>
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<tr>
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<td>12</td>
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<td>13</td>
<td><em>Corynebacterium xerosis</em> JQ267757</td>
<td>482</td>
<td>95</td>
</tr>
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</table>

*Out of 13 total barns.*

### TABLE 3 IgG specific for *S. rectivirgula* in plasma of dairy barn workers compared to that in plasma of controls

<table>
<thead>
<tr>
<th>Population (n)</th>
<th>No. of subjects (%) with each immune response intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Control subjects (35)</td>
<td>30 (85.7)</td>
</tr>
<tr>
<td>Workers (29)</td>
<td>18 (62.1)</td>
</tr>
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</table>

*Frequency distribution between workers and control subjects is significantly different (P = 0.0427).*
cantly higher production of antigen-specific IgG in the plasma of workers compared to that of the control subjects (Table 3). Because the amount of plasma \textit{S. rectivirgula}-specific IgG is used as one of the diagnostic tools for farmer’s lung (29), dairy barn workers are still at risk for developing this airborne disease.

\textbf{Conclusion}. We demonstrated that dairy barn bioaerosols contain high concentrations of various species of methanogenic archaea and bacteria, especially \textit{M. smithii}, an immunogenic archaean species, and \textit{S. rectivirgula}, which is the causal agent of farmer’s lung. These results improve our knowledge of aerobiological burden in this work environment. This study reveals the consistency in the results obtained from three different air samplers. Dairy barn workers are exposed to \textit{S. rectivirgula} despite the recommendations for hay management. A strong similarity between data from various air samplers is observed. This is the first study on dairy barn bioaerosols using molecular biology, which allowed the discovery of airborne archaea that may be involved in the etiology of agriculture-related respiratory diseases.

\textbf{ACKNOWLEDGMENTS}

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