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Airborne Microflora in Quebec Dairy Farms: Lack of Effect of Bacterial Hay Preservatives

Pediococcus pentosaceus is a lactic-acid producing bacterium inoculated in hay to prevent hay deterioration. This study sought to verify the effect of this treatment on the barn microenvironment. Air samples were obtained from 19 barns using bacterial hay treatment and from 18 control barns with six-stage Andersen samplers and all-glass impingers. Appropriate culture media were used for the recovery and identification of microorganisms. Endotoxins were measured with chromogenic *Limulus* amoebocyte lysate assay. Median values (respectively for treated and untreated hay barns) were: 5.28×10^5 and 3.84×10^5 colony-forming units (CFU)/m³ for total bacteria; 3.18×10^6 and 4.5×10^6 CFU/m³ for molds; 1.36×10^3 and 1.74×10^3 endotoxin units/m³ for endotoxin levels; and 1.03×10^3 and 3.00×10^3 CFU/m³ for *Saccharopolyspora rectivirgula*. No viable *P. pentosaceus* were recovered. The presence of *S. rectivirgula*, the causative agent for farmer's lung, was not influenced by the hay treatment. Since no significant difference was observed in any of the airborne contaminants, this type of hay treatment probably does not protect farmers from the respiratory effect of ambient microbial contaminants.

Keywords: aerobiology; agriculture; lactic acid bacteria; farmer's lung

Biological particles are almost always present in the air. Their numbers and types, especially in indoor environments, depend on the microflora of the source material, the degree of disturbance, and the amount of air movement or ventilation. Stored products, such as hay, are often responsible for large numbers of airborne microorganisms. The microflora of stored hay depend on the storage conditions, especially water content and temperature.[1,2] Farm workers are exposed to high levels of contaminants such as bacteria, molds, endotoxins, and plant and animal products that are present in large quantities: in the farm environment, mold spore concentration may reach 10^{11} colony-forming units (CFU)/m³,[3] which is very high compared with the house environment where mold spores typically reach a maximum of 6000 CFU/m³.[4] In dairy barns hay and straw are important sources of airborne molds and bacteria, which rapidly colonize these products, especially when stored with large water contents.[1,2] The inhalation of large concentrations of airborne microorganisms found in these barns can lead to a variety of respiratory problems[3] ranging from bronchitis,[5,6] rhinitis, asthma, and allergy[5] to an allergic alveolitis called farmer's lung.[7,8]

The cloud of dust produced when moldy hay is handled may contain more than 10^9 fungal spores/m³.[9] These spores and mold mycelia are associated with organic dust toxic syndrome.[10,11]

Endotoxins are important components of gram negative bacteria. They are present in sufficient amounts in agricultural environments to cause immunotoxic response.[12] Exposure to endotoxin concentrations of 0.3 µg/m³[13] or 100 endotoxin units (EU) per cubic meter[14] can cause a decrease in lung function.

In wet climates such as that of Quebec, optimal drying of hay before baling is often impossible, and it is sometimes preferable to bale hay at high moisture levels to prevent leaf loss. Hay is therefore often baled with more water than is safe for storage. When hay is stored too moist (>20% moisture), microbial activity becomes very high. This activity results in hay deterioration caused by molds (mostly *Aspergillus* sp.)[2] and in an increase in temperature due to the metabolism of microorganisms.[15] The amount and nature of microorganisms in stored hay is a good

indicator of its moisture level at baling. Baled hay can be dried by hay dryers when bales are small and the hay is loosely packed. However, farmers now frequently harvest their hay in large tightly packed round bales that cannot be dried by this technique.

High temperature ($>50°C$) leads to the growth of thermophilic species such as actinomycetes (e.g., *Saccharopolyspora rectivirgula, Thermoactinomyces vulgaris*) and molds (*A. fumigatus*).[2] These microorganisms are responsible for a form of hypersensitivity pneumonitis called farmer's lung disease.[16]

Various strategies are used by farmers to minimize microbial contamination of their work environment. Among these is the treatment of hay at baling with acids,[17] ammonia,[18] or viable bacterial cultures.[18,19] The use of chemicals is losing popularity because of their corrosive nature and their toxic potential to animals[20] and to humans when inhaled. Lactic-acid producing bacterial cultures such as *Pediococcus pentosaceus* are frequently used. Their growth is claimed to stop microbial growth when hay is stored at moisture levels ranging from 20 to 30% (supplier's recommendations). However, in experimental hay treatment, *P. pentosaceus* inoculum failed to grow in hay samples stored at less than 35% moisture, and such treatment did not significantly modify hay preservation.[19]

Hay treatments have mostly been studied from an agricultural point of view, most of the important studies looking at their effects on hay conservation,[18] nutritive value (dry matter loss),[15,17,18,21] and animal health and lactation.[22] One study was performed in 1983 that looked at the effect of ammonium propionate hay treatment on airborne microorganisms.[23] No reported studies have looked at the effects of bacterial hay preservatives on air quality. The authors felt that such a study was important, since almost half of the farmers questioned (13/30) said that they were using hay preservatives for lung health protection.[24]

It was of particular interest to verify the effect of *P. pentosaceus* treatment on *S. rectivirgula* airborne concentrations, since this bacterium is the most frequently cited causative agent of farmer's lung in Quebec and many other countries.[7,16] It was also important to verify whether the inoculated bacteria, *P. pentosaceus*, would become an additional airborne microbe. Since this bacterium is inoculated at about 500,000 CFU/g of hay, even if it did not grow, it could become airborne. The authors verified in a previous study the effect of nasal instillation of *P. pentosaceus* total extracts in a mouse model of hypersensitivity pneumonitis and found it as potentially antigenic as *S. rectivirgula*.[25]

The main objective of the current study was to compare the total culturable mesophilic bacterial counts, total culturable molds, endotoxins, culturable *S. rectivirgula*, and *P. pentosaceus* from barns where treatment was used with that of control barns where no hay preservative had been added.

Since little is known about microbial air contamination in North American dairy farms,[26] and most of the previous studies described the air microflora in European countries, the aim of this study was also to describe the air contamination caused by hay handling in Eastern Canadian dairy barns.

MATERIALS AND METHOD

Farm Selection

Nineteen farms were chosen on the basis of their use of *P. pentosaceus* hay treatment for at least 2 years. The owners of these farms were asked to find a control farm within 3 miles from theirs (to minimize climatic differences) where hay preservatives had never been used. Control farms had to be similar in type (i.e.,

dairy), haying techniques, size of the barn, and number of cattle. A total of 37 farms were selected. The authors were unable to find a suitable control farm for one of the treated barns. The data presented in this report are therefore from 37 farms: 19 farms where *P. pentosaceus* was used (treated) and 18 control farms (untreated). The farms were visited during two consecutive winters (1993–1994 and 1994–1995). Precipitation was within the normal range over the period of baling in 1993 (325 mm) but was greater than the average for this period in 1994 (488 mm). These data were obtained from Quebec City Airport's annual meteorological summary, *Environment Canada*.

Culture Media

Tryptic-soy agar (TSA) (Difco, Detroit, Mich.) supplemented with cycloheximide (500 mg/L) was used for the isolation of total mesophilic bacteria and *S. rectivirgula*; MRS (DeMan, Rogosa and Sharpe; Oxoid, Basingstoke, UK) supplemented with cycloheximide 500 mg/L was used for the isolation of *P. pentosaceus*. Sabouraud dextrose agar (Difco) and Czapek solution agar (Difco) supplemented with chloramphenicol (50 mg/L) were used for molds culture. Less nutritious media were used (Czapek solution agar) to decrease the proliferation of fast-growing colonies and to improve detection of smallest colonies and their identification (*Cladosporium* sp.). All media were autoclaved at 121°C for 10 minutes.

Air Sampling

Sampling was started as hay was being fed to the animals. Two types of air samplers were used simultaneously: two six-stage Andersen impactors (Grasby Andersen, Atlanta, Ga.) and three all-glass liquid impingers (AGI-30, Ace Glass Inc., Vineland, N.J.). AGI-30 impingers were used to allow dilution of the samples since barn air is known to contain high levels of microorganisms. The Andersen impactors were used at a sampling flow of 28.3 L/min. They were loaded with either TSA to determine the amount of *S. rectivirgula* with a 5-minute sampling, or with MRS agar to determine the amount of *P. pentosaceus* with a 7-minute sampling time. The petri dishes in the Andersen sampler were filled with 45mL of culture media, as recommended.[27] The three AGIs contained 20 mL of sterile saline solution (0.8% NaCl) and used at 12.5 L/min for 8 minutes. These samplers were used to determine total culturable bacteria, total culturable molds, culturable *S. rectivirgula*, *P. pentosaceus*, and endotoxin levels.

Samples Analysis

The Andersen petri dishes were incubated at 52°C for *S. rectivirgula* (TSA) and at 30°C for *P. pentosaceus* (MRS agar).

The samples from the impingers were measured (to evaluate evaporation) and recovered. The impingers were then washed with a volume of sterile saline solution containing 0.075% Tween 80 to increase the sample volume to 30 mL. The final sample contained about 0.03% Tween 80. One milliliter of this 30-mL volume was diluted in 9 mL of sterile saline solution (10^{-1}) and this was repeated to a 10^{-5} dilution. Each sampling suspension (three per farm) was diluted in triplicates. The sixfold dilutions (10^0 to 10^{-5}) were plated on different media appropriate for the microorganism to be studied. For total bacteria and *S. rectivirgula*, TSA was used. MRS agar was used for *P. pentosaceus*. Molds were grown on SDA and Czapek solution agar. All plates were incubated at 30°C except for *S. rectivirgula*, which were incubated at 52°C. The samples from the impingers were frozen for subsequent endotoxin determination. After 60 hours of growth, total bacteria were counted on plates containing between 30 to 300 CFU.

Colonies on TSA incubated at 52°C were counted after 4 to 5 days of growth on Andersen and/or impinger plates according to the concentration found: When *S. rectivirgula* concentrations were low enough to be measured by Andersen sampler, the counts from this impactor were used; when the dishes were overloaded, counts were obtained from the AGI-30. The *S. rectivirgula*-like strains were isolated on TSA at 52°C and kept at 4°C on TSA for further identification. Every colony showing some morphological difference was isolated and identified (or grouped prior to identification). The first isolation was done according to the orange-yellow color, the colony morphology (actinomycete-like), and the growth temperature (52°C). Strains were compared and grouped and then identified.[28,29] The strains were compared with a control strain (ATCC 15347) on the basis of different biochemical reactions: optimal growth temperature; heat; antibiotics and lysozyme resistance; arbutin; esculine; starch; gelatin; adenine; xanthine; hypoxanthine tyrosine; casein and DNA degradation; carbohydrate utilization as sole carbon source; acid production from these sugars; and cell wall analysis.

Molds on SDA were counted after 7 days of incubation. All macroscopically different species were isolated and identified by macroscopic and microscopic analysis. Molds were allowed to grow for 10 days on Czapek agar, counted, isolated, and identified. The counts were performed on plates containing between 5 to 50 CFU/plates.

After 48 hours of incubation, MRS plates from Andersen and/or impinger sampling were observed and *P. pentosaceus* colonies counted. Suspected colonies were isolated (white, brilliant, regular colony morphology, and catalase negative) and identified using API 50-CH system (BioMérieux, Lyon, France).

Endotoxins from impinger samples were measured using the *Limulus* amoebocyte assay chromogenic test (Associates of Cape Cod, Woods Hole, Mass.). Samples were diluted 10 times, twofold in a pyroplate, and the dilution giving an optical density from 0.3 to 0.6 at 405 nm was used for endotoxin determination. Controls were obtained with sterile saline water with which the sterile AGI-30 sampler was washed for a few minutes. This procedure allowed the measurement of the initial contamination of the samples by endotoxins. For technical reasons endotoxin measurement was not performed on the first 11 samples (Barns 1 to 11).

Statistical Analysis

This study specified the following comparisons: barn air microflora with and without *P. pentosaceus* hay treatment. Normality and variance were verified with the Wilks test. According to the data an unpaired Student's t-test or Wilcoxon rank sum test was performed, as appropriate. Graphical representations of total bacteria, endotoxins, *S. rectivirgula*, and mold counts were expressed using points and medians for representative measures, since variance stabilization were not encountered or the empirical distribution function was not symmetric. Mold species (percentage of total mold counts) were expressed using means plus or minus standard deviation.

RESULTS

Total amount of bacteria recovered on TSA at 30°C is shown in Figure 1. The results ranged from 1.30×10^4 to 9.01×10^6 CFU/m³ in farms using *P. pentosaceus* (treated) and from 4.43×10^4 to 3.64×10^6 CFU/m³ in control farms (untreated). No significant difference was observed between treated (median = 5.29×10^5 CFU/m³) and untreated (median = 3.84×10^5 CFU/m³) farms (p=0.94).

FIGURE 1. Total culturable bacteria (CFU/m³) measured on *P. pentosaceus*-treated and untreated (control) farms (— = median)
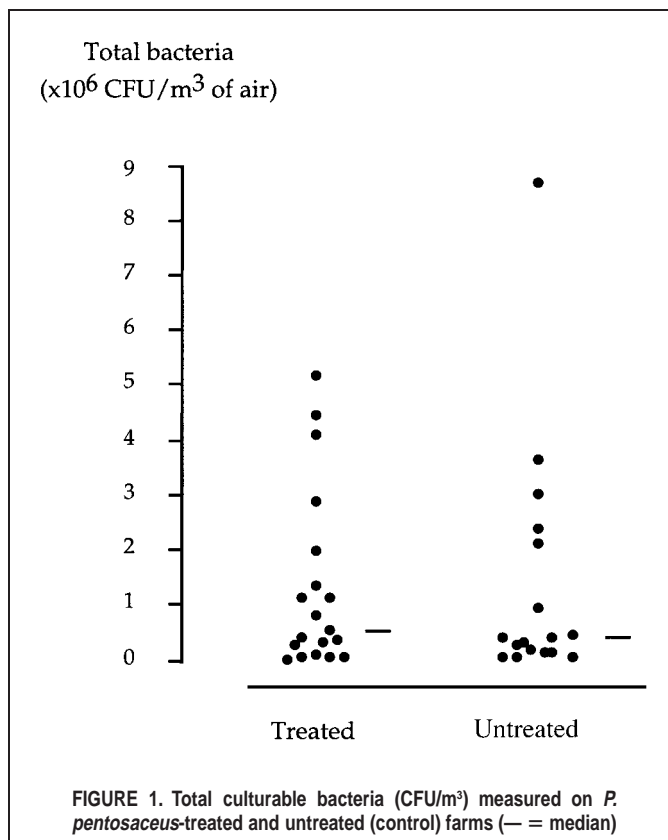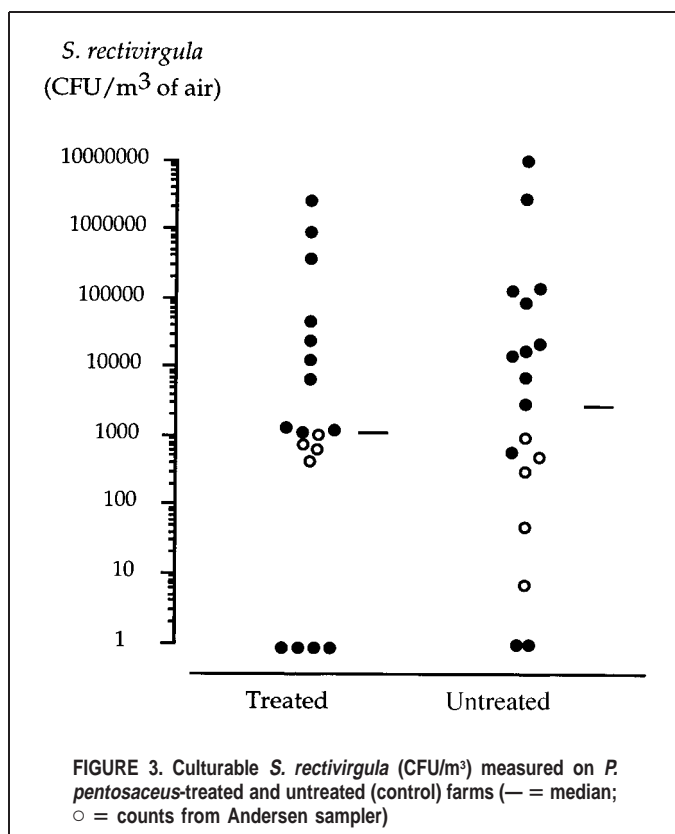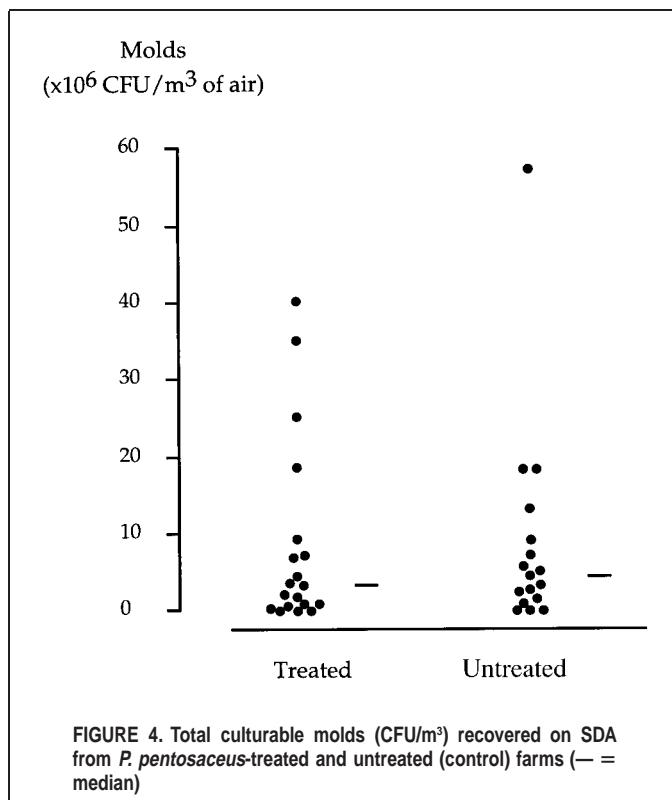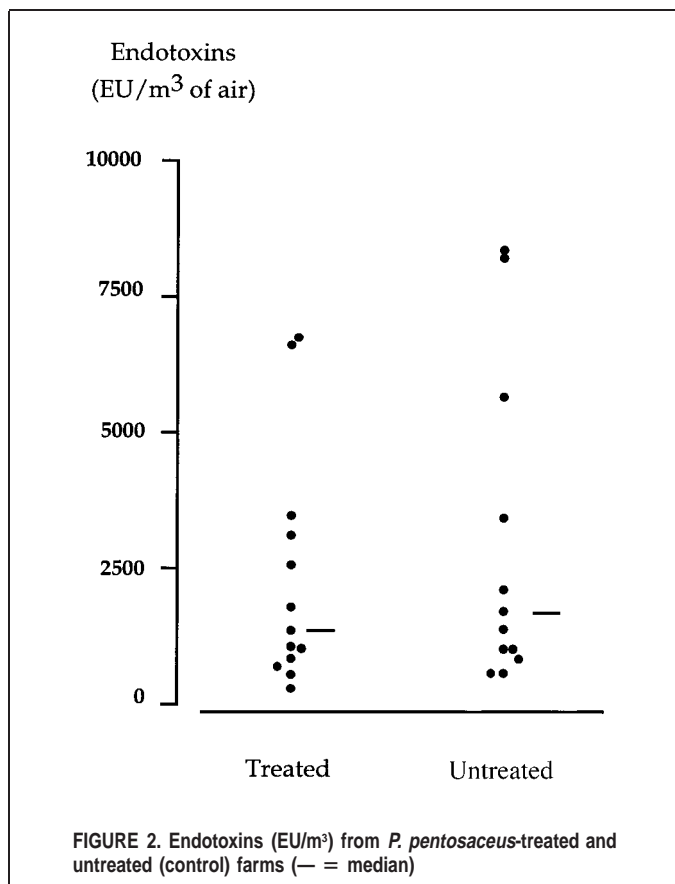
Endotoxin amounts ranged from 349 to 6.82×10^3 (median = 1.36×10^3) EU/m³ for treated and from 615 to 8.35×10^3 (median = 1.74×10^3) EU/m³ in untreated farms (p= 0.43) (Figure 2). (10 EU = 1 ng *Escherichia coli* standard LPS). Note that 11 points are missing on this figure: measurement was not performed on Barns 1 to 11.

The number of *S. rectivirgula* recovered colonies ranged from 0 to 2.36×10^6 (median = 1.02×10^3) and to 3.1×10^7 (median = 3.0×10^3 CFU/m³ for treated and untreated farms, respectively (Figure 3). Among the values presented on this figure, 10 were from Andersen counts. The number of Andersen values was the same in each group. The averages in Andersen counts were much lower than those of the impingers (2×10^6 versus 535 CFU/m³). The *S. rectivirgula* values were not significantly different between the two groups (p=0.98).

Larger amounts of molds were recovered from SDA (median of 3.18×10^6 CFU/m³ for treated and 4.54×10^6 CFU/m³ for untreated farms) (Figure 4) than from Czapek agar (median = 3.15×10^5 CFU/m³ for treated and 2.43×10^5 CFU/m³ for untreated farms) (data not shown) but no significant difference was observed between treated and untreated farms (p=0.98 with SDA and 0.47 with Czapek). The species of molds recovered on both media were the same in treated and untreated farms. Most molds recovered were from the *A. glaucus* group: the median was 57% of total molds in treated farms (on SDA) and 39% of total molds in untreated farms (on SDA) (Figure 5) (difference not significant, p=0.35). Mostly the same mold populations were observed on Czapek agar (the majority being *Aspergillus*) but this media allowed the recovery of *Fusarium* sp., *Helminthosporium* sp., *Scedosporium apiospermum*, some *Aspergillus* species (*A. versicolor*, *A. nidulans*) and slow-growing colonies such as *Cladosporium* sp. (Figure 6).

No living *P. pentosaceus* was detected in the air of the studied barns.

Endotoxins
(EU/m³ of air)

FIGURE 2. Endotoxins (EU/m³) from *P. pentosaceus*-treated and untreated (control) farms (— = median)

Molds
(x10⁶ CFU/m³ of air)

FIGURE 4. Total culturable molds (CFU/m³) recovered on SDA from *P. pentosaceus*-treated and untreated (control) farms (— = median)
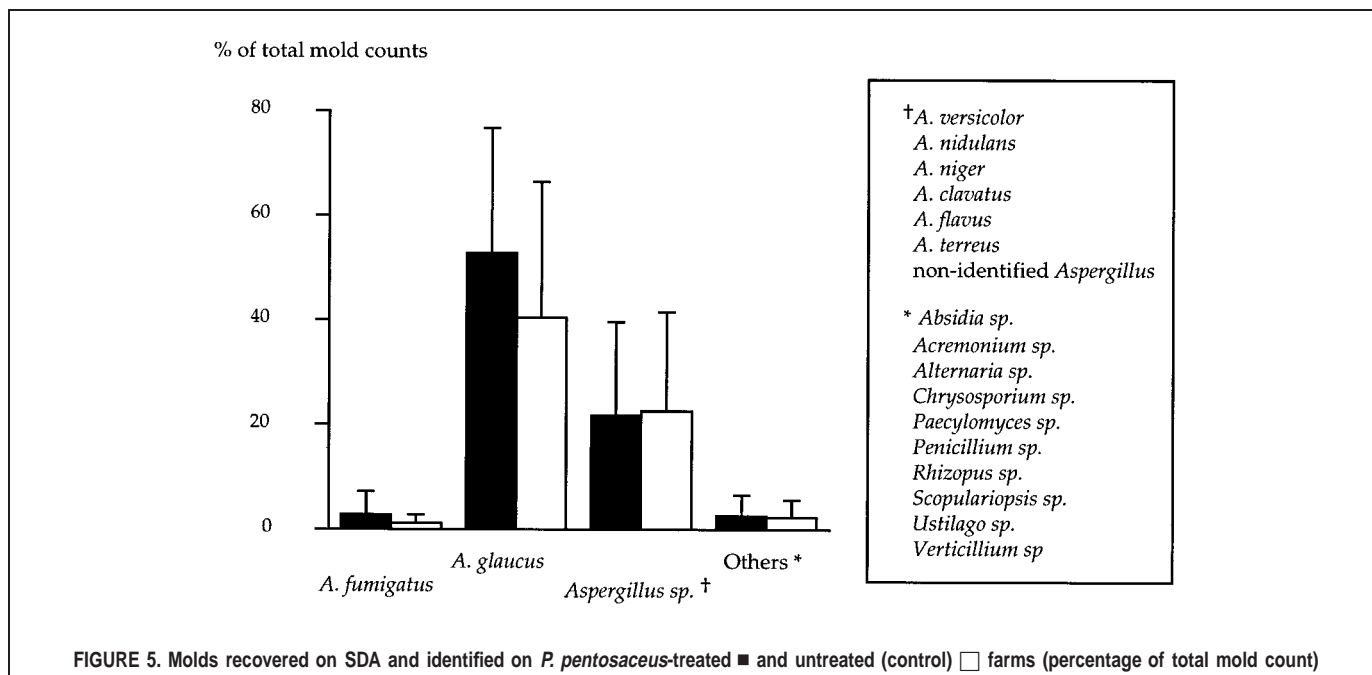
DISCUSSION

All barns were studied between November and April, months when animals are kept and fed in the barn, the doors and windows are closed, and ventilation is kept at its minimum to conserve heat during this cold season. Air sampling took place several months after baling. Hay heating and microbial activity occur in the first days after baling and the microbial content remains stable for weeks thereafter.[1] Therefore, no important changes in microflora would be expected between November and April.

Results of this study show that total culturable airborne bacterial counts were similar in treated and untreated farms. The counts were very high and consistent with previous studies where the number of viable bacteria reported ranged from 10^4 to 10^7 CFU/m³.[3] Most importantly, airborne *S. rectivirgula* concentrations were not altered by *P. pentosaceus* treatment.

The most important molds found in barn air were from the *A. glaucus* (*Eurotium*) group. These molds are responsible for hay deterioration when it is stored at about 30% moisture levels,[2,30] and they were previously found in great numbers in barn air.[31] *A. glaucus* molds typically grow when hay is molding but not heating.[30] It is thus not surprising to find great quantities of these molds in barn air when hay is handled. Thermoresistant fungi such as *A. fumigatus* were detected in almost all the barns. However, total counts of these colonies are hard to determine because these molds grow very fast even at 30°C and when present, are often confluent. Czapek solution agar allowed a better recovery of *A. nidulans* and *Cladosporium* sp. colonies.

Mesophilic fungi may be found in all environments. In the present study molds such as *Alternaria* sp. and *Cladosporium* sp. were found in great amounts in some barns. They characterize the microflora from good quality hay.[30,31] They were found in the same amount on treated and untreated farms; therefore, the treatment does not alter the presence of these fungi.

S. rectivirgula
(CFU/m³ of air)

FIGURE 3. Culturable *S. rectivirgula* (CFU/m³) measured on *P. pentosaceus*-treated and untreated (control) farms (— = median; ○ = counts from Andersen sampler)

% of total mold counts

FIGURE 5. Molds recovered on SDA and identified on *P. pentosaceus*-treated ■ and untreated (control) ☐ farms (percentage of total mold count)

The amounts of endotoxin detected in the current report are about 1500 EU/m³ (average of the two median values). The measured values are 7 to 167 times higher than the guideline range for humans, calculated at about 50 EU/m³[32] over an 8-hour exposure period. Concentrations found in this study are those usually associated with febrile reactions and irritation of the upper respiratory tract. These high levels of endotoxins were, however, very surprising. Poultry houses and swine buildings are typically associated with high endotoxin levels.[3] Such high endotoxin concentrations are probably due to the time when samplings were performed. These levels, found during hay handling and vigorous shaking of litter and straw, probably do not represent the whole-day exposure, but the peak of contamination. The farmers, therefore, are probably exposed to such levels only when they are handling the hay. Endotoxins could be responsible for the previously reported febrile reactions in dairy farmers.[33] Treatment of hay with *P. pentosaceus* is not able to reduce air contamination by endotoxins.

The authors did not evaluate the effect of *P. pentosaceus* treatment on hay quality and therefore cannot comment on the efficiency of this hay inoculant to prevent hay molding and heating. However, they and others previously verified the inefficiency of *P. pentosaceus* or other bacterial hay treatment in preventing hay deterioration in laboratory conditions.[18,19] These experiments failed to show an affect on hay conservation under various moisture levels. The bacteria did not grow when moisture was at 20, 25, or 30% but grew at 35% moisture; in no condition did it improve conservation. Even when *P. pentosaceus* proliferated in 35% moisture hay, all culturable inoculated bacteria disappeared within 2 months; samplings at the farms were done 6 to 8 months after hay treatment. This may help in understanding why no viable *P. pentosaceus* was detected in barn air.

The winters when sampling was done followed a very rainy (1994) and a normal summer (1993). It is probable that some hay was baled at relatively high moisture levels (higher than 20%). The suppliers recommend the use of the hay treatment between 20 and 30% moisture. Therefore, the lack of effect of hay treatment cannot be explained by the utilization of the product in unfavorable conditions. However, the presence of great amounts of *S. rectivirgula* in the barns confirms that heating had occurred in high moisture hay.

It was previously shown that if a chemical treatment is unevenly distributed in hay, mold will grow in untreated regions and will spread to treated parts of the baled hay.[34] This observation may be applied to bacterial treatment. It was also found that molds from the *A. glaucus* group both tolerate and metabolize propionic acid.[35] It is possible that molds from this group can tolerate lactic acid produced by *P. pentosaceus*. Furthermore, since *P. pentosaceus* is not likely to grow in hay stored at moisture levels that allow *A. glaucus* molds (and other xerotolerant molds that can grow at 20% moisture), the inhibition of *Aspergillus* by lactic acid is probably not very important.
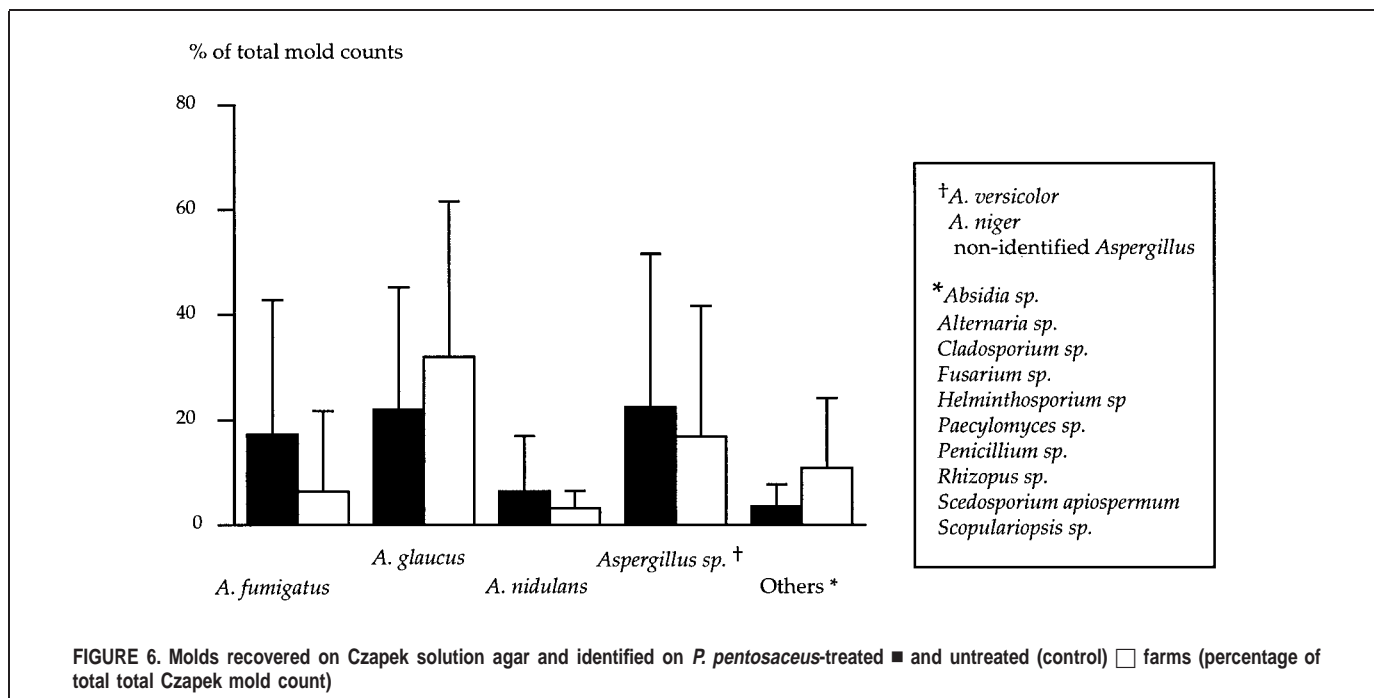
Sampling viable microorganisms is known to underestimate the amount of microbial antigens and particles present. However, this method was valid to determine any effect of *P. pentosaceus* treatment; both treated and control farms were studied by the same procedure. It is not known whether nonviable *P. pentosaceus* antigens were present.

CONCLUSION

This study showed the lack of effect of *P. pentosaceus* hay treatment on barn air microflora. There were no significant differences in the nature or the amount of airborne microbial contaminants (bacteria, fungi, endotoxins, and *S. rectivirgula*) when *P. pentosaceus* was used as hay treatment.

Barn air in Quebec is highly contaminated with molds and bacteria. The nature of these is similar to that previously reported. Potentially harmful levels of endotoxin can also be found in dairy barns at the time of hay handling.

Since *P. pentosaceus* treatment does not change the amount and the nature of airborne microorganisms including *S. rectivirgula*, it is unlikely that its use will decrease risks for farmer's lung disease or other respiratory diseases related to barn air microbial contaminants.

% of total mold counts

FIGURE 6. Molds recovered on Czapek solution agar and identified on *P. pentosaceus*-treated ■ and untreated (control) ☐ farms (percentage of total total Czapek mold count)

Although no viable *P. pentosaceus* was found, this study does not rule out the possibility that seeded bacterial products could become airborne and add another potentially harmful allergen in dairy barns. Further studies are required to verify whether farmers who use *P. pentosaceus* develop antibodies or allergies to this bacteria.

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APPLIED STUDIES