

Proinflammatory effect of *Pediococcus pentosaceus*, a bacterium used as hay preservative

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Proinflammatory effect of Pediococcus pentosaceus, a bacterium used as hay preservative. C. Duchaine, E. Israël-Assayag, M. Fournier, Y. Cormier. ©ERS Journals Ltd 1996.

ABSTRACT: Bacterial cultures, such as *Pediococcus pentosaceus*, are used to treat hay with the objective of preventing hay heating and moulding, and thus, the development of the microbial growth which causes farmer's lung. The aim of this study was to investigate whether such bacterial cultures have the potential to induce a pulmonary inflammatory response.

Mice were instilled 3 days·week⁻¹ for 3 weeks with either saline or nonviable preparations of *P. pentosaceus*, *Saccharopolyspora rectivirgula*, *Lactococcus lactis* (control bacteria) or with the combinations of *S. rectivirgula* and *P. pentosaceus*.

P. pentosaceus induced a significant inflammatory response in the lung which was similar to that produced by *S. rectivirgula*. *L. lactis* produced a response of a lower intensity. The total number of cells in bronchoalveolar lavage were: *S. rectivirgula*: 6.4×10^5 cells·mL⁻¹; *P. pentosaceus*: 4.3×10^5 cells·mL⁻¹; *S. rectivirgula* + *P. pentosaceus*: 5.4×10^5 cells·mL⁻¹, *L. lactis*: 6.8×10^5 cells·mL⁻¹ and saline group 3.7×10^4 cells·mL⁻¹. The lung index was higher in *S. rectivirgula* + *P. pentosaceus* and *P. pentosaceus* groups than in *S. rectivirgula*, *L. lactis* and saline groups. The quantity of specific immunoglobulin G and A (IgG and IgA) to *P. pentosaceus* and *L. lactis* levels (in the blood and/or lavage fluid) were similar to those against *S. rectivirgula*.

In mice, *P. pentosaceus* has the potential to induce a similar inflammatory response in the lung as *S. rectivirgula*, which is the most common antigen responsible for farmer's lung disease in Quebec. Further studies are needed to verify whether farmers can develop farmer's lung or other lung responses to this new potential antigen.

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Farmer's lung is a well-described hypersensitivity pneumonitis, most frequently caused by thermophilic actinomycetes, such as *Saccharopolyspora rectivirgula* [1], moulds or a mixture of microbial species which grow in hay that heats when stored in conditions that are too humid. In an attempt to reduce the risk of farmer's lung, products are available commercially to treat hay in order to prevent it moulding and heating and, thus, encouraging microbial growth. These products include acids (lactic, acetic, propionic) and lyophilized viable lactic acid-producing bacterial cultures (*Lactobacillus* sp., *Pediococcus* sp.). When inoculated, bacteria are guaranteed by the manufacturer to be present in large amounts in the treated hay, at least 500,000 colony forming units (cfu)·g⁻¹ of hay. These bacteria are nontoxic when fed to animals.

Numerous bacteria and fungi become airborne when hay (especially if poorly preserved) is fed in closed barns. Barn air contains large quantities of actinomycetes, fungi and bacteria [2–4]. It is probable that the bacteria used to treat hay could also become airborne. No studies have been reported on the potential risk to the lung when these bacteria are inhaled. Since these airborne bacteria

would be inhaled by farmers who have used them to treat their hay, it is important to ensure that they are innocuous to the lung. These bacteria are not pathogens, but it could be argued that they might induce hypersensitivity reactions, such as farmer's lung.

Animal models are frequently used to verify the potential of microbial products to induce lung inflammation. We and others have studied lung response in C57Bl/6 mice instilled with *S. rectivirgula* [5, 6]. In the current study, this animal model was used to evaluate lung response to *Pediococcus pentosaceus*, a bacterium frequently used to treat hay in Quebec. We were interested in studying these bacteria alone and in combination with other microorganisms found in dairy barns in Canada. This latter aspect is important, since a new bacterium could be inoffensive by itself and still present a potential hazard as a co-factor with other environmental contaminants. The objective of this study was, therefore, to compare the inflammatory potential of *P. pentosaceus* with that of *S. rectivirgula* and another metabolically similar (lactic acid producing) bacterium, *Lactococcus lactis*, which was used here as a control.

Methods

Microbial preparations

P. pentosaceus, isolated from the commercial hay preservative, was purified and identified by using the API 50CH system (Biomérieux, Montreal, Canada). Fresh cultures of *S. rectivirgula* T-150 (ATCC 15347), *P. pentosaceus*, and *L. lactis* (previously *Streptococcus lactis*, ATCC 11454) were obtained: *S. rectivirgula* was grown in tryptic soy broth (Difco) at 52°C for 4 days, *P. pentosaceus* and *L. lactis* were grown in de Man, Rogosa and Sharpe (MRS) broth [7] (Oxford/Unipath, Basingstoke, UK) at 30°C for 2 days. Nonviable endotoxin-free preparations of each of these bacterial cultures were prepared as described previously [8]. Briefly, bacteria were cultured in a shaking water bath, harvested by centrifugation, washed with sterile water and disrupted with a Braun cell homogenizer. After lyophilization, the product was solubilized at 5 mg·mL⁻¹ in saline water. Aliquots of this suspension were instilled in mice as described below.

Animals and sensitization

Fifty pathogen-free C57Bl/6 female mice, weighing 20 g, were used (Charles River Inc., St Constant, Quebec, Canada). Groups of animals were kept in separate plastic cages. Under light isoflurane-induced anaesthesia (Anaquest, Mississauga, Ontario, Canada), mice were nasally instilled on three successive days each week for 3 weeks. Five groups of 10 mice were studied: saline; *S. rectivirgula*; *P. pentosaceus*; *L. lactis* and *S. rectivirgula* + *P. pentosaceus* groups. These mice were instilled with either 50 µL of 5 mg·mL⁻¹ (250 µg total) of nonviable preparations of the respective bacteria or with 50 µL of sterile saline solution. Different animals from each group were used to measure lung index and histology, or for bronchoalveolar lavage (BAL).

Lung index and histopathology analyses

Four days after receiving their last instillation, five mice from each group were anaesthetized with isoflurane, weighed and sacrificed by cervical dislocation after blood sampling by retro-orbital puncture. The lungs were removed, trimmed of extraneous tissue, rinsed and weighed. Lung index was determined by the equation [9]:

$$\text{Lung index} = \frac{(\text{lung weight/body weight})_{\text{test animal}}}{(\text{lung weight/body weight})_{\text{naive animal (average)}}}$$

The lungs were then fixed in Bouin's solution, embedded in paraffin, cut into 5 µm sections and stained with haematoxylin and eosin for histopathological analysis.

Histopathology was evaluated by a medical pathologist, without knowledge of treatment. Each lung was scored for inflammatory cell infiltrates, focal lesions with or without epithelioid cells, lymphoid hyperplasia and lymphoid agglomerates. A total score was given for inflammatory lesions and an average score was obtained for each group of mice: +=light inflammation; ++=moderate inflammation; +++=substantial inflammation; ++++=very substantial inflammation.

BAL and determination of cell populations

After blood sampling, five mice from each group were sacrificed 4 days after their last instillation. For the lavage, the tracheas were immediately cleared and cannulated (20 G plastic catheter). The lungs were washed with three aliquots of 1 mL sterile physiological saline solution. The lavage fluid was centrifuged at 1,200 rpm for 10 min. The supernatants were kept at -20°C until antibody determination, and cell pellets were resuspended in 500 µL of saline solution. Twenty five microlitres of the resuspended cells was mixed 1:2 with crystal violet (total cells count), and the same dilution was made with trypan blue (cells viability). About 200,000 cells were subjected to Diff-Quik staining (Baxter-Canlab) [10] and nonspecific esterase colouration [11]. The different cell populations were determined by counting 600 cells.

Antibody analysis

Blood samples were centrifuged at 1,000 rpm for 10 min, and sera were frozen at -20°C until analysis. For each antigen used, Nunc MaxiSorp Immuno plates were coated with 100 µL of 250 µg·mL⁻¹ of the antigen preparation and incubated overnight at 4°C. The next morning, plates were washed and saturated with 1% bovine serum albumin to block residual protein binding sites of the plate. Specific antibodies (to each antigen studied) and cross-reaction (to all antigens) in serum and in lavage fluid (see below for BAL) were determined by enzyme-linked immunosorbent assay (ELISA). A titration was first made to determine the best dilution to minimize cross-reaction and zero level and to maximize specific reaction. Dilutions used were 1:1000 for immunoglobulin G (IgG) measurement in serum, 1:2 for immunoglobulin A (IgA) and 1:4 for IgG in lavage fluid. Duplicates of diluted samples were incubated in coated plates, and anti-mouse IgG and IgA coupled to peroxidase with ortho-phenylenediamine (OPD) as the substrate were used. Absorbance was read at 490 nm.

Statistical analysis

Comparisons with the control group were performed using Dunnett's technique. A p-value of <0.05 was considered significant.

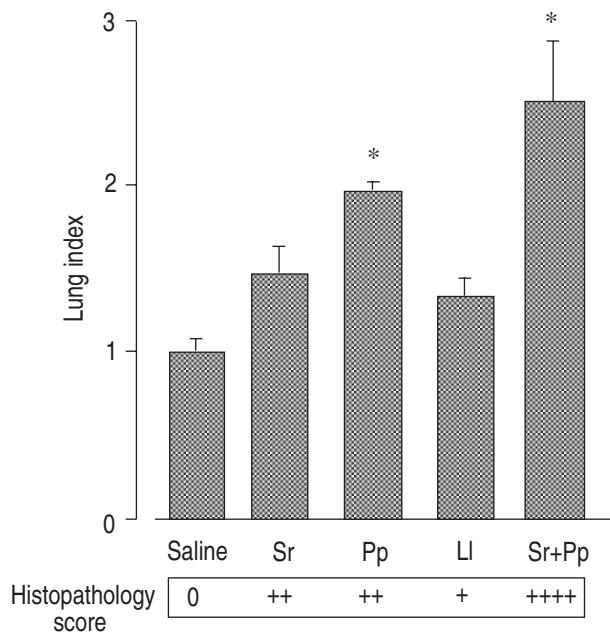


Fig. 1. – Lung indices (mean±SEM) and gradation of the intensity of all lung lesions observed on histopathology for all groups of mice. *Pediococcus pentosaceus* (Pp) induced more lung inflammation/oedema than *Saccharopolyspora rectivirgula* (Sr) alone. In contrast, *Lactococcus lactis* (Ll) did not increase lung index. Histopathological analyses were in accordance with lung indices. *: $p < 0.05$, compared to saline group. +: light inflammation; ++: moderate inflammation; ++++: very substantial inflammation

Results

Pulmonary inflammation was characterized by oedema, cellular infiltrates and an increased lung index. The increase of lung index following instillation of *P. pentosaceus* (1.95 ± 0.10) was greater than that following instillation of *S. rectivirgula* (1.46 ± 0.17) ($p < 0.05$). Instillation of both *P. pentosaceus* and *S. rectivirgula* further increased this index (2.49 ± 0.35) ($p < 0.05$). In contrast, *L. lactis* did not induce a significant increase in lung index (fig. 1). The histopathological analysis was in accordance with lung indices. The main pathological findings were peribronchial and peri-vascular cellular infiltrates, with a corresponding decrease of the bronchial and alveolar space. The average pathological score was similar for *S. rectivirgula* and *P. pentosaceus* (++) and higher for the *S. rectivirgula* + *P. pentosaceus* group (++++). In the *L. lactis* group, very light cell infiltrates were observed (+) (figs. 1 and 2).

The volume of BAL fluid recovery (about 2.5 mL for each mouse) was similar for all groups (data not shown). With all antigens used, the total number of cells increased compared to control group (fig. 3), while the percentage of macrophages, neutrophils and lymphocytes remained similar (table 1). No eosinophils were observed.

Blood and lavage specific IgG were detected against all antigens tested and specific IgA were present in lavage fluid (fig. 4). No cross-reactions were seen between different antigens either in the blood or in lavage fluid (data not shown).

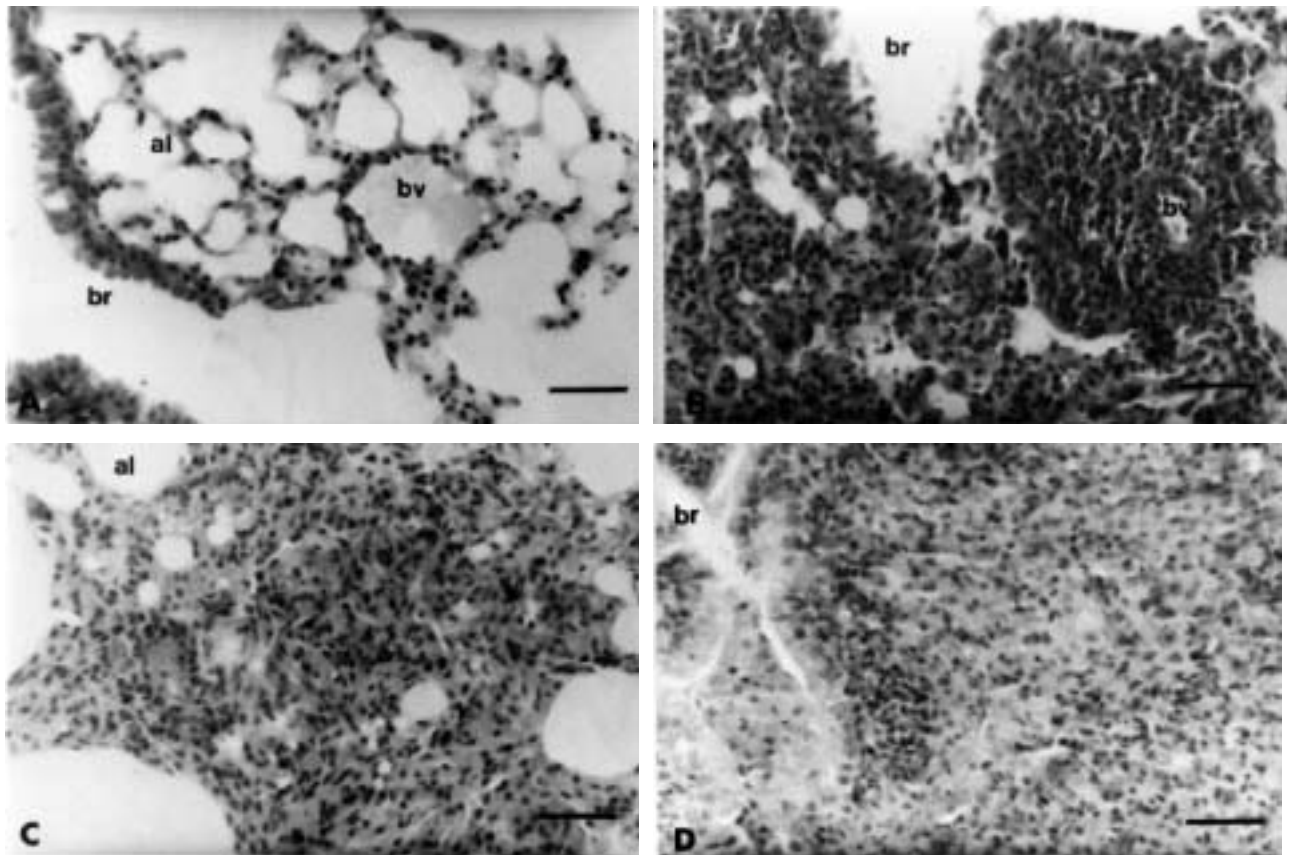


Fig. 2. – a) Photomicrograph of normal lung (saline). b–d) Photomicrographs of lung histology showing typical lesions found in: b) *Saccharopolyspora rectivirgula*; c) *Pediococcus pentosaceus*; d) *S. rectivirgula* + *P. pentosaceus*. In b–d note the conspicuous cellular inflammatory infiltration. al: alveoli; bv: blood vessel; br: bronchi. Scale bar=25 μ m, original magnification $\times 400$.

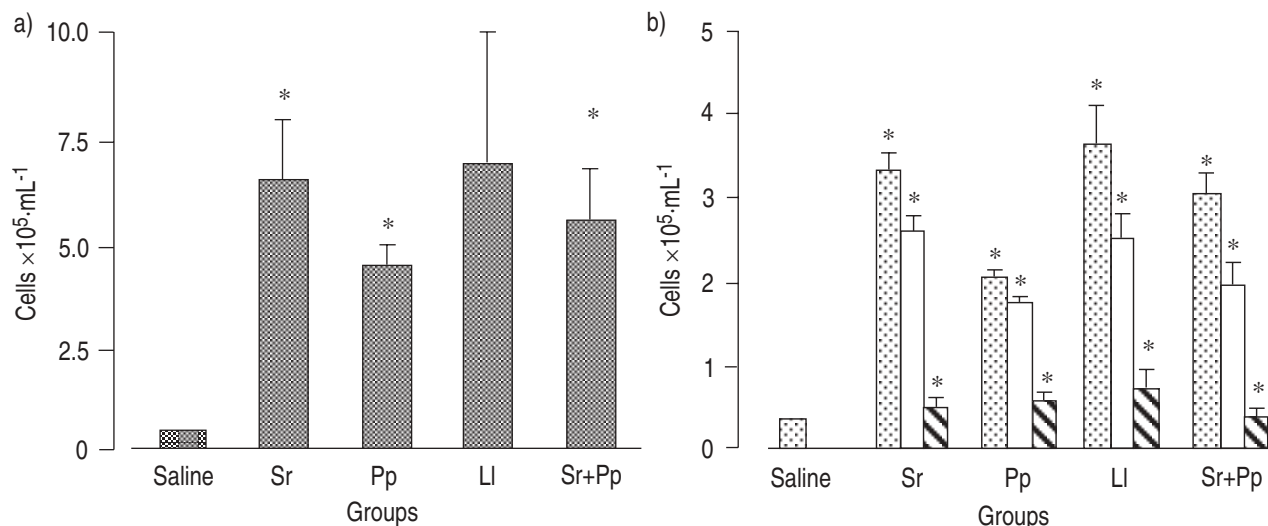


Fig. 3. – a) Concentration of total lavage cells; and b) *Saccharopolyspora rectivirgula* (Sr), *Pediococcus pentosaceus* (Pp) and Sr+Pp increased the total number of lavage cells. The increase with *Lactococcus lactis* (LI) was not significant. ▨ : macrophages; ▤ : lymphocytes; □ : neutrophils. *: $p < 0.05$, compared to saline group.

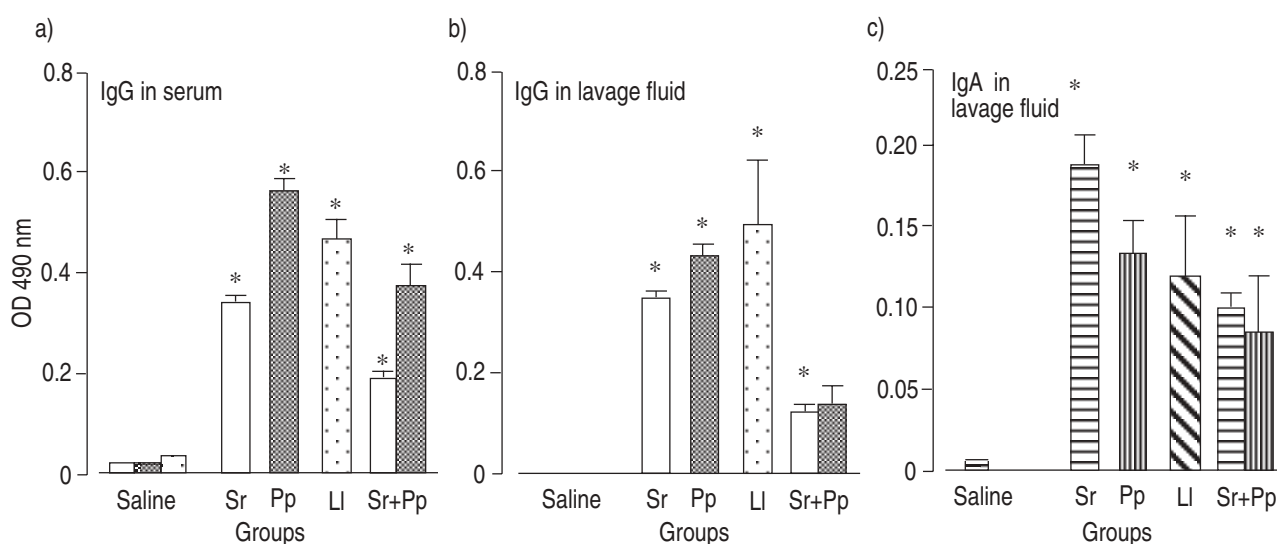


Fig. 4. – a) Specific immunoglobulin G (IgG) in serum; b) specific IgG in lavage fluid and c) specific immunoglobulin A (IgA) in lavage fluid, expressed in optical density (OD) at 490 nm. Values are expressed as mean \pm SEM. Specific immunoglobulin against all antigens used were detected in serum and in lavage fluid. All three specific antibodies were tested in the five groups and no cross-reaction was observed (data not shown). □ : IgG anti-*Saccharopolyspora rectivirgula* (Sr); ▤ : IgG anti-*Pediococcus pentosaceus* (Pp); ▨ : IgG anti-*Lactococcus lactis* (LI); ▩ : IgA anti-Sr; ▧ : IgA anti-Pp; ▦ : IgA anti-LI. *: $p < 0.05$ compared to saline group.

Table 1. – Percentages of cell differentials in saline (control), *S. rectivirgula* (Sr), *P. pentosaceus* (Pp), *L. lactis* (LI) and *S. rectivirgula* + *P. pentosaceus* (Sr + Pp) groups

	Saline	Sr	Pp	LI	Sr+Pp
Macrophage	96.5 \pm 0.6	51.6 \pm 3.3*	46.7 \pm 2.5*	52.8 \pm 6.6*	55.1 \pm 5.1*
Lymphocytes	2.1 \pm 0.9	40.8 \pm 2.7*	40.1 \pm 1.7*	36.7 \pm 4.1*	35.6 \pm 4.4*
Neutrophils	1.4 \pm 0.4	7.5 \pm 2.4*	13.2 \pm 1.9*	10.5 \pm 3.3*	6.5 \pm 1.8*

Values are mean \pm SEM. *: $p < 0.05$ compared with saline groups.

Discussion

The results of this study show that *P. pentosaceus*, a bacterium frequently used as a hay preservative with the objective of preventing mould and bacterial growth and heating, can induce a similar pulmonary response in mice as *S. rectivirgula*, the most common antigen responsible for farmer's lung disease in Quebec.

Simple parameters were used to evaluate the effect of each bacterium on the lung. We believe this approach was justified, since this animal model has previously been well described for *S. rectivirgula* [5, 12]. The objective of this study was to compare *P. pentosaceus* to another related microorganism and to *S. rectivirgula*, not to look at the specific mechanisms involved. Blood and lavage specific IgG and IgA, lung index, pathology and

lavage cells were evaluated. The variables analysed were chosen because they are either routinely used in our mouse model [11] or because they are important clinical parameters of farmer's lung disease [13, 14]. All these parameters gave similar results, pointing to the same conclusion, *i.e.* that *P. pentosaceus* is as proinflammatory as *S. rectivirgula*. Since specific antibodies (IgG and IgA) were produced against *P. pentosaceus* as against *S. rectivirgula*, and since pulmonary inflammatory cells and lung index similarly increased in both groups, mice seem to develop similar inflammatory and humoral immune response to both bacterial preparations.

Although the number of lavage cells recovered in *L. lactis* treated mice did not reach statistical significance, some animals had a high cell yield. The large variability in the responses to this antigen can explain this lack of statistical significance. However, other parameters (lung index and histopathology) support a lower antigenic potential of *L. lactis*. We do not know whether *L. lactis* can cause farmer's lung: this was not the objective of the current study. In Quebec, 40% of patients with farmer's lung have negative precipitins to *S. rectivirgula*, *Aspergillus fumigatus* and *Thermoactinomyces*. These patients could very well have farmer's lung to other antigens.

Different non-pathogenic microorganisms (Rhizopus, Phanerochaete, Penicillium) have previously been tested in an animal model (guinea-pigs) using aerosols exposure [15]. That study showed that the development of inflammation is not confined to one or a few microorganisms, but could be expected in any highly contaminated environment [15]. In this context, treating hay with a bacterial preservative could, in itself, add yet another potentially harmful allergen to the air. Treatment with *P. pentosaceus* could, however, still be advantageous if the added bacteria significantly prevents growth of other microorganisms, such as moulds and thermophilic actinomycetes. This would depend on the relative level of bacteria present. However, if the total number of bacteria is increased by the addition of *P. pentosaceus*, this treatment could increase the risk for farmer's lung. Also, if treated hay still contains *S. rectivirgula*, this combination of bacteria could be an added risk factor for the disease.

One must be careful in extrapolating results from an animal model to human conditions. Also, in the barn environment, the relative proportions of each of these two bacteria would not likely be equal, as in the current study. How different combinations of the two bacteria would interfere with or modify the *S. rectivirgula* response is unknown. Further studies are needed to verify whether *P. pentosaceus* treatment effectively prevents *S. rectivirgula* growth, whether *P. pentosaceus* is present in barn air, and whether farmers develop antibodies and or farmer's lung to this added potential antigen.

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References

1. Pepys J, Jenkins PA, Festenstein GN, Gregory PH, Lacey ME, Skinner FA. Farmer's lung: thermophilic actinomycetes as a source of "farmer's lung hay" antigen. *Lancet* 1963; ii: 607-611.
2. Eduard W, Lacey J, Karlsson K, Palmgren U, Strom G, Blomquist G. Evaluation of methods for enumerating microorganisms in filter samples from highly contaminated environments. *Am Ind Hyg Assoc J* 1990; 51: 427-436.
3. Kotimaa MA, Terho EO, Husman K. Airborne moulds and actinomycetes in the work environment of farmers. *Eur J Respir Dis* 1987; 152 (Suppl.): 91-100.
4. Louhelainen M, Kangas J, Husman K, Terho EO. Total concentrations of dust in the air during farm work. *Eur J Respir Dis*. 1987; 152 (Suppl.): 73-79.
5. Cormier Y, Tremblay GM, Fournier M, Israël-Assayag E. Long-term enhancement of lung response to *Saccharopolyspora rectivirgula*. *Am J Respir Crit Care Med* 1994; 149: 490-494.
6. Schulyer M, Gott K, Haley P. Experimental murine hypersensitivity pneumonitis. *Cellular Immunology* 1991; 136: 303-317.
7. De Man JC, Rogosa JC, Sharpe ME. A medium for the cultivation of lactobacilli. *J Appl Bacteriol* 1960; 23: 130-135.
8. Schulyer M, Schmitt D, Steinberg D. Hypersensitivity pneumonitis in strain II guinea-pigs. I. Histological features. *Int Arch Allergy Appl Immunol* 1982; 68:108-111.
9. Wilson BD, Stermick JL, Yoshizawa Y, Katzenstein A, Moore VL. Experimental murine hypersensitivity pneumonitis: mutagenic control and influence by genes within 1-B subregion of the H-2 complex. *J Immunol* 1982; 129: 2160-2163.
10. Laviolette M, Carreau M, Coulombe R. Bronchoalveolar lavage cell differential on microscope glass cover: a simple and accurate technique. *Am Rev Respir Dis* 1988; 138: 451-457.
11. Li CY, Lam KW, Yam LT. Esterases in human leukocytes. *J Histochem Cytochem* 1973; 21: 1-12.
12. Denis M, Cormier Y, Laviolette M, Ghadirian E. T-cells in hypersensitivity pneumonitis: effects of *in vivo* depletion of T-cells in a mouse model. *Am J Respir Cell Mol Biol* 1992; 6: 183-189.
13. Cormier Y, Laviolette M. Farmer's lung. *Semin Respir Med* 1993; 14: 31-37.
14. Roberts RC, Wenzel FJ, Emanuel DA. Serum immunoglobulin levels in farmer's lung disease. *J Allergy Clin Immunol* 1973; 52: 297-302.
15. Fogelmark B, Lacey J, Rylander R. Experimental allergic alveolitis after exposure to different microorganisms. *Int J Exp Path* 1991; 72: 387-395.