Propionic acid bacteria as protective cultures in fermented milks and breads

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Abstract — Strains of lactic acid bacteria (LAB) and propionic acid bacteria (PAB) were screened as biopreservatives against food spoilage yeasts, molds and Bacillus spp. singly and in combination. A combination of Lactobacillus rhamnosus strain LC705 and Propionibacterium freudenreichii ssp. shermanii strain JS was found to be the most active against yeasts, molds and Bacillus spp. The combination was tested for its activity against yeasts and molds in different food applications and the best results were obtained in fermented milks and in bakery products. An initial level of $10^7$ cells.g$^{-1}$ fermented milk product and a level of $10^8$ cells.g$^{-1}$ sour dough were found to be effective against yeasts and Bacillus spp. Technology for the use of the combination in different applications was developed. © Inra/Elsevier, Paris.

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1. INTRODUCTION

Traditionally, propionic acid bacteria (PAB) are known for their ability to convert lactate to propionate, acetate and CO$_2$, which is responsible for the formation of eyes in Swiss-type cheese [10]. A potential new role for PAB in food preservation has been recently introduced. 'Microgard™' is a well known PAB based biopreservative in which inhibitory activity has been associated with diacetyl, propionic, acetic and lactic acid and to a heat-stable 700–Da peptide [5]. 'Microgard™' inhibits most Gram-negative bacteria and some fungi [3]. Bacteriocins of PAB have been studied and reviewed recently by Barefood and Grinstead [4]. Other uses of PAB in the control of undesirable micro-organisms have been reported by Odame-Darkwah and Marshall [13] who showed that the bread spoilage bacterium, Bacillus pumilus, was inhibited by P. freudenreichii ssp. shermanii. Propionic acid and its salts are accepted as preservatives for industrial use in bread manufacture because of their inhibitory activity against mold and Bacillus ssp. [8]. In addition to food applications, PAB and propionic acid have also been used as preservatives in silage [9].

Most of the research and development work in biopreservation has concentrated on lactic acid bacteria (LAB). These produce various antimicrobial compounds such as organic acids, H$_2$O$_2$, diacetyl, bacteriocins and bacteriocin-like substances. Studies on bacteriocin-producing LAB were reviewed by De Vuyst and Vandamme [7]. The activity of LAB cultures and their bacteriocin production has been studied in various food systems and some bacteriocins (nisin and pediocin) are already used in food preservation. Unfortunately, Gram-negative bacteria, yeasts and molds are not inhibited by LAB bacteriocins, although yeasts and molds are frequently the cause of hygienic problems in fermented milk products and bread.

The objective of this work was to develop a protective culture which would improve the shelf life of fermented milks and bread, and which would replace chemical additives such as sorbic and acetic acids in different applications. Further aims were to optimize the amount of antagonistic culture needed for the inhibition of undesirable microbes and to develop a technology for using the culture in fermented milks and in wheat bread.

2. MATERIALS AND METHODS

2.1. Bacterial strains and preparation of the protective culture

Lactobacillus rhamnosus LC705 (DSM 7061) and Propionibacterium freudenreichii ssp. shermanii JS (DSM 7067) were from the Valio culture collection. The protective culture, called Bioprofit, was produced by growing both strains together in a medium consisting of 5% (w/v) whey permeate (Valio Ltd, Helsinki, Finland), 2% (w/v) casein hydrolysate (Valio Ltd), and 1% (w/v) yeast extract (LAB M, Bury, UK) in a BIOSTAT (B. Braun, Melsungen, Germany) fermentor connected to a microfiltration system (Millipore, Bedford, USA) for 48 h at 30 °C at pH 5.5. The protective culture was used in applications as a fresh concentrated culture.

Commercial starters for quark (Probat 505) and for yogurt (V2) were obtained from Wiesby GmbH, Niebull, Germany. The DL-culture for quark was composed of undefined multiple species of Leu. lactis ssp. cremoris, Le. lactis ssp. lactis, Le. lactis ssp. lactis biovar. diacetylactis and Leuconostoc mesenteroides ssp. cremoris (Probat 505). The yogurt starter consisted of different strains of Streptococcus thermophilus and Lactobacillus bulgaricus.

Rhodotorula rubra RHO and Pichia quillermondii PQ were both isolated from spoiled fermented milk products and were from the Valio culture collection. They were grown in Bacto YM-broth (Difco Laboratories, Detroit, USA) at 25 °C for 2 d before use. Bacillus subtilis P.2.94 and Bacillus licheniformis P.1.94 were isolated from aropy wheat bread. Bacillus strains were sub-cultured twice in BHI-broth (LAB M) for 24 h at 37 °C before use. Spores were obtained by growing these Bacillus strains on nutrient agar
plates fortified with 0.003 % (w/v) MnSO₄·4H₂O and 0.025 % (w/v) KH₂PO₄, aerobically at 37 °C for 48 h. The spores were collected by centrifugation at 4 500 g for 10 min at 4 °C, washed twice with sterile water and then resuspended in sterile water (50 mL) to form a stock solution. The stock solution was heated at 80 °C for 10 min before storage at 4 °C. The stock solution contained 9 × 10⁸ spores·mL⁻¹.

2.2. Manufacture of quark

Quark was manufactured according to the thermo-quarg process [15] using Probat 505 as starter. The protective culture concentrate was mixed with the fresh fermented quark mass, after manufacture at levels of either 0.01 % (v/v) or 0.1 % (v/v). Controls were prepared without and with calcium sorbate which was suspended in water and added to the fresh fermented quark mass at a final concentration of 0.06 % (w/v). The quark masses were then artificially contaminated with R. rubra RHO before packing. Yeasts, molds, LAB and PAB were analyzed once per week during storage at 6 °C for 5 weeks and organic acids were analyzed at the beginning and after 3 weeks of storage at 6 °C.

2.3. Manufacture of yogurt

Control yogurt was fermented with a commercial yogurt culture (V2). Test yogurt was prepared as for control yogurt with the addition of the protective culture to give an initial level of > 10⁷ cells of protective culture·g⁻¹ yogurt. The protective culture was added at the same time as the starter. Yogurt was fermented at 42 °C for about 3 h until the pH reached pH 4.5. Strawberry jam was contaminated either with R. rubra RHO or P. quillermondi PQ at concentrations of 100–500 cells·g⁻¹. Contaminated jam was added to yogurt at 14 % (v/v). Basic starters, LAB, PAB and yeasts were enumerated once per week during storage at 6 °C for 4 weeks and organic acids were analyzed at the beginning and after 3 weeks of storage at 6 °C.

2.4. Manufacture of wheat bread with sour dough

Bread was made by a natural sour dough process without intentional addition of starter. Test bread was manufactured by mixing equal amounts of wheat flour and water with the protective culture to a homogenous mass, which was then fermented for either 4, 10 or 20 h at 30 °C. This sour dough was added at different levels to the final dough. Control bread was manufactured for each fermentation time in the same way but without the protective culture. The final dough was made of 1 700–2 100 g wheat flour, 170 g liquid salt (26 % w/w), 170 g liquid yeast (67 % w/v), 96 g butter, 500–800 g water, 172 g liquid sugar (77 % w/v) and 400–1 200 g sour dough. An inoculum of 3 × 10⁸ B. subtilis and B. licheniformis spores g⁻¹ dough was added to each dough. The final doughs were baked at 220 °C for 30 min. Breads were stored at 20–22 °C at an RH of < 20 % at 28–30 °C and at an RH of 70 % for 6 d. The bread was analyzed for Bacillus spp., organic acids, pH and total titratable acidity (TTA) during storage for 6 d.

2.5. Microbiological analyses

Samples were analysed for lactobacilli on MRS-agar (LAB M, Bury, UK) and for L. rhamnosus LC705 on MRS supplemented with 0.005 % (w/v) of vancomycin (MRSV) (Sigma Chemical Co., St Louis, USA), for propionic acid bacteria on sodium lactate agar (YEL) with the addition of 1 % (w/v) of β-glycerophosphate (Merck, Darmstadt, Germany), for yeasts and molds on YCG (LAB M) and for Bacillus spp. on Phenol Red Egg Yolk Polymyxin agar (LAB M) by spread-plating. MRS and MRSV plates were incubated anaerobically at 37 °C for 3 d, YEL-plates were incubated anaerobically at 30 °C for 7 d, YCG plates were incubated aerobically at 25 °C for 3 d and Bacillus cereus plates aerobically at 37 °C for 20–24 h.

2.6. Physico-chemical analyses

pH and total titratable acidity (TTA) were analysed using a modified standard procedure [1] by suspending 10 g bread in 2 mL of acetoin to break the dough structure and 98 mL of water and titrating the sample with 0.1 N NaOH to pH 8.5 with a titrator (Mettler DL 20, GWB, Zurich, Switzerland). Lactic acid concentrations were determined enzymatically (Boehringer Mannheim, Mannheim, Germany). Propionic and acetic acids were determined by gas chromatography (Perkin-Elmer Sigma 3B GC coupled to a AS 300 auto-
sampler) using Chromosorb WAW 80/100 as the stationary phase (3 m × 2 mm, id.) [16]. Ethanol and diacetyl were determined by capillary gas chromatography (Carlo Erba GC 6000 Vega coupled to a HS 250 autosampler) using a 30 m × 0.32 mm (id.) SPB-1 silica column (4.0 mm film, Supelco) as the stationary phase [17]. Benzoic acid was determined by isocratic reversed phase liquid chromatography using a C-18 column (3.2 × 250 mm, packed with 5 mm Spherisorb ODS) equipped with an UV-detector (Waters, Milford, USA) according to [2].

3. RESULTS

3.1. Inhibition of yeasts in fermented milk products

Cell numbers of L. rhamnosus LC705 and P. freudenreichii ssp. shermanii JS in the protective culture concentrate were 2 × 10^{10} cfu-mL^{-1} and 2 × 10^{10} cfu-mL^{-1}, respectively. A level of 0.1 % (v/v) of the protective culture equivalent to 2 × 10^{7} cells-g^{-1} of both strains, inhibited the growth of R. rubra RHO whereas 0.01 % (v/v), equivalent to levels of 2 × 10^{6} cells of both strains g^{-1}, had no effect on the growth of the contaminant (figure 1). The initial level of yeast was 2 to 3 × 10^{2} cells-g^{-1} quark which reached 8 × 10^{6} cells-g^{-1} in the control and in test quark containing 0.01 % (v/v) of the protective culture after storage at 6 °C for 5 weeks. In test quark containing 0.1 % (v/v) of the protective culture, the yeast counts remained at a level of ~1 × 10^{2} cells-g^{-1} during storage at 6 °C for 5 weeks. The organisms in the protective culture did not grow during the storage (data not shown). The cell numbers of L. rhamnosus LC705 and P. freudenreichii ssp. shermanii JS, respectively. The strains of the protective culture did not grow in the yogurt during the manufacture and the cell numbers remained at ~10^{7} cfu g^{-1} yogurt during storage for 4 weeks at 6 °C (data not shown).

3.2. Physico-chemical and sensory properties of fermented milks

No difference in the concentrations of lactic acid was obtained in either the quark or the yogurt, manufactured with or without the protective culture after storage at 6 °C for 3 weeks. In yogurt, the concentration of propionic acid manufactured with the protective culture was 2 mg·100g^{-1} whereas the concentration of propionic acid in the control yogurt was less than the detection limit (< 0.5 mg·100g^{-1}). In quark manufactured with the protective culture, no propionic acid was formed by P. freudenreichii ssp. shermanii JS as the concentration of propionic acid was less than the detection limit. The concentration of acetic acid was higher in quark and in yogurt to which the protective culture had been added than in the controls. The concentration of diacetyl was higher in both quark and yogurt containing the protective culture than in the controls. A high amount of ethanol was found in control yogurt, which was probably produced by P. quilermondii PQ (table 1 and figure 2).

3.3. Effective process for wheat bread

The initial levels of L. rhamnosus LC705 and P. freudenreichii ssp. shermanii JS were of 1 × 10^{8} and 3 × 10^{8} cells-g^{-1} of sour-dough, respectively. Their numbers did not increase during the fermentation of the sour dough for 4 or 10 h but after fermentation for 20 h, the numbers of L. rhamnosus LC705 increased by one log-unit while those of
Figure 1. Growth of *Rhodotorula rubra* RHO at 6 ℃ in quark to which 0.01 % (v/v) protective culture (○), 0.1 % (v/v) protective culture (●), or 0.06 % (w/v) calcium sorbate (□) was added after manufacture, and control without any additive (■).

Figure 1. Croissance de *Rhodotorula rubra* RHO à 6 ℃ dans du fromage blanc additionné après fabrication de (○) 0,01 % (v/v) de culture protectrice ; (●) 0,1 % (v/v) de culture protectrice ; (□) 0,06 % (w/v) sorbate de calcium ; (■) contrôle sans additif.

Figure 2. Growth of *Pichia quiliermondii* PQ at 6 ℃ in yogurt fermented without (●) or with (○) 0.1 % (v/v) of the protective culture and of *Rhodotorula rubra* RHO in yogurt fermented without (■) or with (□) 0.1 % (v/v) of the protective culture.

Figure 2. Croissance de *Pichia quiliermondii* PQ à 6 ℃ dans un yaourt fermenté sans (●) ou avec addition (○) de 0,1 % (v/v) de culture protectrice et de *Rhodotorula rubra* RHO dans un yaourt fermenté sans (■) ou avec addition (□) de 0,1 (v/v) de culture protectrice.
Table I. Levels of organic acids, diacetyl and ethanol in quark and yogurt manufactured with basic starter and with the addition of 0.1 % protective culture after storage for 3 weeks at 6 °C. (Control = product without protective culture; protective culture = product with 0.1 % protective culture [v/v]).

<table>
<thead>
<tr>
<th></th>
<th>Quark</th>
<th>Yogurt</th>
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<tr>
<td></td>
<td>Control</td>
<td>Protective culture</td>
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<tr>
<td>Lactic acid, %</td>
<td>0.72</td>
<td>0.73</td>
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<tr>
<td>Propionic acid, mg·100g⁻¹</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
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<tr>
<td>Acetic acid, mg·100g⁻¹</td>
<td>38.0</td>
<td>64.0</td>
</tr>
<tr>
<td>Diacetyl, mg·kg⁻¹</td>
<td>0.6</td>
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</tr>
<tr>
<td>Ethanol, mg·kg⁻¹</td>
<td>10.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Benzoic acid, mg·kg⁻¹</td>
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<td>nt</td>
</tr>
</tbody>
</table>

nt: not tested / nt : non déterminé.

P. freudenreichii ssp. shermanii JS did not (data not shown). The duration of the fermentation of sour dough with the protective culture had an effect on the inhibition of Bacillus spp. in the bread. No growth of Bacillus spp. was observed in bread manufactured with 10 % of sour dough fermented for 10 or 20 h with the protective culture, whereas Bacillus spp. started to grow after 2 d of storage at 20–22 °C in bread manufactured with 10 % of sour dough fermented for 4 h with the protective culture and in the control bread (figure 3). Similar results were obtained with 20 % sour dough. The vol-

Figure 3. Growth of Bacillus spp. at 20–22 °C for 6 d in wheat bread made with 10 % of sour dough which was fermented with the protective culture for 4, 10 or 20 h. Control bread was made without the protective culture (PC = protective culture; c = control).

Figure 3. Dénombrement de Bacillus à 20–22 °C pendant 6 j dans du pain réalisé avec 10 % de pâte acide fermentée par la culture protectrice pendant 4, 10 ou 20 h. Le pain de contrôle était réalisé sans culture protectrice (PC = culture protectrice ; c = contrôle).
Propionibacteria as biopreservatives

Volume of sour dough added to the dough had an effect on the Bacillus spp. counts especially in warm and moist conditions. Bread with 10 to 20% of sour dough fermented with the protective culture for 10 h inhibited the growth of Bacillus spp. totally, whereas in the control bread, the number of Bacillus reached a level of $10^4$ g$^{-1}$ after 6 d of storage at 20–22 °C at an RH of < 20% (figure 4a). In warm and moist conditions (28–30 °C; RH of 70%) Bacillus spp. were inhibited for at least 6 d in bread made with 15 to 20% of sour dough fermented for 10 h with the protective culture, whereas in the control bread, Bacillus spp. reached a level of $10^4$ cells·g$^{-1}$ after 2 d. Bread made with 10% of sour dough showed high counts of Bacillus spp. after 3 d storage (figure 4b).

Figure 4. Growth of Bacillus spp. in wheat bread during (A) storage for 6 d at 20–22 °C at an RH of < 20% and (B) at 28–30 °C at an RH of 70% for 6 d. Breads were made by adding 10, 15 or 20% of sour dough fermented with or without the protective culture for 10 h. Control bread was made without the protective culture (PC = protective culture).

Figure 4. Dénombrement de Bacillus dans du pain conservé : (A) 6 j à 20–22 °C à une humidité relative < 20% ; (B) 6 j à 28–30 °C à une humidité relative de 70%. Les pains étaient réalisés avec addition à la pâte de 10, 15 ou 20% de pâte acide fermentée avec ou sans la culture protectrice pendant 10 h. Le pain de contrôle était réalisé sans culture protectrice (PC = culture protectrice).
3.4. Physico-chemical quality of bread

The duration of the fermentation time of the sour dough had an effect on the pH and on the lactic and propionic acid but not the acetic acid concentrations of bread manufactured with the protective culture (table II). It was observed that the longer the fermentation time, the higher the concentration of lactic acid and the lower the pH of the bread manufactured with the protective culture. The level of propionic acid did not increase beyond 10 h fermentation. No differences in the concentrations of acetic acid were obtained in either test bread or control bread, except for the lower concentration of acetic acid (15 mg·100g⁻¹) in the test bread fermented for 20 h than in other breads (18–19 mg·100g⁻¹).

4. DISCUSSION

Sorbitic, benzoic and acetic acids are used widely in the food industry as preservatives because of their antimicrobial activity against various bacteria, yeasts and molds [6]. Our studies focused on replacing these additives with an antagonistic culture containing L. rhamnosus LC705 and P. freudenreichii ssp. shermanii JS. The combined effect of strains of the protective culture against spoilage yeasts and molds [12] and Bacillus ssp. was stronger than the effect of either culture alone (data not shown) presumably due to a synergistic effect of the different metabolites of this mixed culture. The mechanism and the mode of inhibitory action has not been characterized.

The shelf life of fermented milks was prolonged by initial levels of $2 \times 10^7$ cells of

Table II. The effect of duration of fermentation of the sour dough on pH, total titratable acidity (TTA) and concentrations of organic acids in wheat bread. Bread was made with sour dough fermented with an initial level of both L. rhamnosus LC705 and P. freudenreichii ssp. shermanii JS 1–3 × 10⁸ cells·g⁻¹ dough for 4, 10 or 20 h by adding 20 % of the sour dough to the final dough. Control bread was fermented without protective culture for 4, 10 or 20 h by adding 10 % sour dough to the dough.

<table>
<thead>
<tr>
<th>Fermentation time</th>
<th>Lactic acid, %</th>
<th>Propionic acid, mg·100g⁻¹</th>
<th>Acetic acid, mg·100g⁻¹</th>
<th>pH</th>
<th>TTA, mL</th>
</tr>
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<tbody>
<tr>
<td>4 h</td>
<td>Control PC</td>
<td>Control PC</td>
<td>Control PC</td>
<td>Control PC</td>
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<tr>
<td></td>
<td>&lt;0.04 0.04</td>
<td>&lt;0.04 0.04</td>
<td>1.0 4.0</td>
<td>19.0 19.0</td>
<td>5.5 5.5</td>
</tr>
<tr>
<td>10 h</td>
<td>Control PC</td>
<td>&lt;0.04 0.17</td>
<td>1.0 5.0</td>
<td>19.0 19.0</td>
<td>5.5 4.8</td>
</tr>
<tr>
<td>20 h</td>
<td>Control PC</td>
<td>&lt;0.04 0.23</td>
<td>1.0 5.0</td>
<td>19.0 15.0</td>
<td>5.5 4.6</td>
</tr>
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</table>

PC = protective culture / PC = culture protectrice.
both *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS-g-1 product. The cell numbers of *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* did not increase during the storage of fermented milks at 6 °C for 4 weeks, but the protective strains continued to metabolize as the concentrations of diacetyl and acetic acid in quark and the concentrations of diacetyl, propionic and acetic acids in yogurt increased during storage. In a production scale test of quark, the protective culture at a level of $2 \times 10^7$ cells·g⁻¹ inhibited the growth of molds (data not shown). The sensory quality of this product was superior to the control product due to the production of diacetyl from citrate by the protective culture. The protective culture did not interfere with the basic starters in yogurt as the cell counts of *S. thermophilus* and *L. bulgaricus* were similar in both the control yogurt and in the yogurt manufactured with the protective culture.

Inhibition of yeasts and molds by the protective culture cannot be based on pH and acids alone. Propionic acid and its salts are primarily inhibitory to molds and *Bacillus* spp. at concentrations of 0.1–5 % [8]. In our studies, using the protective culture, the concentration of propionic acid reached 0.002 % (yogurt), < 0.005 % (quark) and 0.005 % (wheat bread). Acetic acid is effective against yeasts and bacteria at concentrations of 0.4–0.8 % (w/v) [8], while, the concentrations in the present study were 0.06 % (w/v) in quark and < 0.02 % (w/v) in bread. Concentrations of diacetyl in fermented milks made with the protective culture were below the inhibitory concentration of 300 mg·kg⁻¹ [5]. Concentrations of lactic acid were 0.78–0.94 % in fermented milks prepared with or without the protective culture. Some reports [5, 11] suggest that the formation of benzoic acid by lactic acid bacteria may be partially responsible for inhibition of yeasts but according to our results, the level of benzoic acid in yogurt did not increase with the protective culture.

The optimal sour dough process was developed with the protective culture of LC705 and JS. The initial level of $1-3 \times 10^8$ of both *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS g⁻¹ sour dough with a fermentation time of 10 h and with the addition of over 10 % to the dough improved the shelf life of the wheat bread. Inhibition of *Bacillus* spp. in wheat bread may be partially explained by lower pH and higher amounts of lactic acid in test bread compared to control bread. In addition, the sensory quality of the test bread was optimal. The pH of the wheat bread made with sour dough is optimal around pH 5 while, below pH 4.2, the product is considered too acidic [14]. The growth of *Bacillus* species causing ropiness in wheat bread was found to be dependent on temperature and moisture of the environment and was faster at an RH of 70 % at 28–30 °C than at an RH of < 20 % at 20–22 °C.

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