Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Research International 38 (2005) 597–604
FOOD RESEARCH INTERNATIONAL
www.elsevier.com/locate/foodres

Probiotic bacteria as adjunct starters: influence of the addition methodology on their survival in a semi-hard Argentinean cheese

C.V. Bergamini [1], E.R. Hynes [2], A. Quiberoni [2], V.B. Suárez, C.A. Zalazar *,[2]

Instituto de Lactología Industrial, Facultad de Ingeniería Química (Universidad Nacional del Litoral), Santiago del Estero 2829, S3000AOM Santa Fe, Argentina

Received 16 November 2004; accepted 25 November 2004

Abstract

Two strains of probiotic bacteria, one of *Lactobacillus acidophilus* and the other of *Lactobacillus paracasei* subsp. *paracasei*, were tested as adjunct cultures in cheese-making experiments, in order to assess their viability during cheese-making and ripening. The adjunct culture was added to cheese-making milk following two different methodologies: as a lyophilized powder dispersed in milk, or within a substrate composed of milk and milk fat. In all cheeses, probiotic bacteria increased a log cycle during cheese-making, and remained almost constant during ripening (60 days), always in higher number than required to meet probiotic standards. Gross composition of the cheeses was not affected by the addition of probiotic bacteria, except for pH value: cheeses with *L. acidophilus* added within the pre-incubated substrate, had lower pH values and were over acidified and crumbly. Direct addition of the probiotic culture was the methodology with the best performance; however the pre-incubation presented some advantages such as an increased population of lactobacilli in the initial inoculum.

Keywords: Probiotic bacteria; Cheese-making; Ripening; Viability

1. Introduction

Functional food is a modified food or food ingredient that provides a health benefit beyond satisfying traditional nutrient requirements, while probiotics are defined as live microbial supplements which beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989; Sanders, 1998).

When a functional food with the addition of probiotic bacteria is developed, it is essential that probiotics maintain their viability not only during the manufacture and storage of the product, but also during the transit of the food through gastrointestinal tract, resisting stomach acidity and bile salts of small intestine (Ziemer & Gibson, 1998). It is not sufficient to establish the concentration of probiotics in a functional food in order to insure their health benefits, because this number varies with the strain and with the food (Ross, Fitzgerald, Collins, & Stanton, 2002). For example, Gardiner et al. (1999) found higher recovery of *Enterococcus faecium* in feces when they were delivered in Cheddar cheese than when they were consumed in yogurt. On the other hand, Donnet-Hughes, Rochat, Serrant, Aeschlimann, and Schiffrin (1999) observed an increase in immune response in humans when a strain of *Lactobacillus johnsonii* was delivered in a dose of 10^9 CFU d^{-1}. However, the same effect was not observed when the dose was 10^8 CFU d^{-1},

* Corresponding author. Tel.: +54 342 4530302; fax: +54 342 4571162.
E-mail address: azalazar@fiqus.unl.edu.ar (C.A. Zalazar).
[1] Doctoral fellow from CONICET(Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina.
[2] Researcher from CONICET, Argentina.

0963-9969/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodres.2004.11.013

even though the probiotic bacteria were similarly recovered in feces after the intake of the two different doses. Nevertheless, 10^7 CFU g^{-1} or mL^{-1} of food, at the moment of the intake, is frequently established as the minimal probiotic population required to impact favorably on the consumer's health (De Vuyst, 2000).

Dairy products have been used as carrier foods for probiotic bacteria, as many of them had already been optimized for survival of lactic cultures (Heller, 2001). Among dairy products, fermented milks (Ziemer & Gibson, 1998) were the most used to deliver the probiotic bacteria, and many papers have been published on this subject (Adhikari, Mustapha, Grün, & Fernando, 2000; Davidson, Duncan, Hackney, Eigel, & Boling, 2000; Sultana et al., 2000; Sun & Griffiths, 2000). However, the low pH of fermented milks can be inadequate for the survival of some probiotic bacterial strains. For example, Vinderola and Reinheimer (2000) found that probiotic population was under the minimum required by legislation in several Argentinean fermented milks. In recent years, it has been suggested that cheese is a more adequate probiotics carrier than fermented milk. The higher pH and fat content, and the solid matrix of cheese, may protect bacteria more efficiently than a fluid environment during the storage of the food and its transit through the human body (Stanton et al., 1998). Vinderola, Prosello, Ghiberto, and Reinheimer (2000) found a higher viability for *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *Lactobacillus casei* in cheese slurry than in hydrochloric solution, when both were tested at pH 3. On the other hand, Gardiner et al. (1999) found a better protective effect in cheese than in yogurt, for a probiotic culture of *E. faecium*.

Different cheeses have been used to deliver different probiotic bacteria (Blanchette & Roy, 1995; Blanchette, Roy, Bélanger, & Gauthier, 1996; Corbo, Albenzio, De Angelis, Sevi, & Gobbetti, 2001; Dinakar & Mistry, 1994; Gardiner et al., 2002; Gobbetti, Corsetti, Smacchi, Zocchetti, & De Angelis, 1998; Gomes, Malcata, Klaver, & Grande, 1995; Lynch, Muir, Banks, McSweeney, & Fox, 1999; O'Riordan & Fitzgerald, 1998; Roy, Mainville, & Mondou, 1997; Stanton et al., 1998; Vinderola et al., 2000). Rogelj, Bogovič Matijašić, Majhenič, and Stojković (2002) working with a semi-hard cheese reported that the population of a probiotic strain of *L. acidophilus* was higher than 10^7 CFU g^{-1} after six months of ripening, and concluded that semi-hard cheeses were suitable food vehicles for probiotic bacteria. In spite of this information on probiotic cheeses, it is important to note that extrapolation to other probiotic strains or food matrices is not always evident. In fact, resistance to salt, acidic and oxidative stress, among other factors, vary from one probiotic strain to another. On the other hand, the cheese-making process determines environmental conditions and slight differences can result in significant changes to the medium (Ross et al., 2002). Moreover, it has been shown that starter and probiotic cultures can interact (Vinderola, Mocchiutti, & Reinheimer, 2002b), but most experiments have been performed on cheese varieties manufactured with mesophilic starter cultures. There is little information about probiotic cheeses manufactured with thermophilic starter cultures, which are the most widely used in Argentina.

Argentina produces approximately 420,000 tonnes of cheese per annum, which places the country among the leading cheese producers in the world. Argentineans are also good cheese consumers (about 10 kg of cheese per year per head) (http://www.cil.org.ar; Fox, 2003). Therefore, the importance of cheese quality for Argentinean economy is considerable, specially taking into account increasing quality standards, more informed consumers, and the fact that most of the current major cheese producers are based in countries with developed economies. However, comparatively little research work has been performed on Argentinean cheese varieties, and further investigation is needed to obtain a better control of the cheese-making and ripening processes, specially if a new factor such as adjunct culture addition is involved. During the last years Argentina produced about 130,000 ton $year^{-1}$ of semi-hard cheeses (http://www.cil.org.ar), among which Pategrás Argentino is the most important variety (Zalazar, Meinardi, & Hynes, 1999). So far, probiotic cultures have not been added to this cheese variety.

The aim of the present work was to determine if preincubation of probiotic bacteria in a substrate composed of milk and milk fat improved their viability in Pategrás Argentino cheese. For that purpose, cheeses with probiotic bacteria added as a freeze-dried powder or as a preincubated culture were made and compared during cheese-making and ripening.

2. Materials and methods

2.1. Cheese-making

Two cheese-making trials were made at pilot plant scale, according to the industrial technology for the semi-hard cheese Pategrás Argentino (Zalazar et al., 1999). The cheese variety was selected as its technology does not include curd treatments such as steady stirring, high temperature cooking, direct salt addition, curd washing, etc., that can impair viability and increase the loss of probiotic bacteria in the whey. Fat matter concentration was fixed at a higher value (3.8%, w/v) than the minimum established by legal regulations for Pategrás cheese (3.0%, w/v) (ANMAT, 1999) in order to standardize fat matter in control and experimental cheeses. In each trial, a different probiotic strain was used as adjunct starter.

Three cheeses were made by cheese-making day: one control cheese and two types of experimental cheeses. Three replicates were performed by trial, i.e. a total of nine cheeses for each tested probiotic strain were made, distributed in three cheese-making days. As for pilot plant characteristics, we made two cheeses simultaneously and the third after they had been molded; the order in which cheeses were made was changed from one cheese-making day to another. In the first type of experimental cheeses (EL), probiotic bacteria were added lyophilized (as freeze-dried powder), while in the second type (EP) the probiotic bacteria were added after pre-incubation in a substrate composed of milk and milk fat. Control cheeses did not contain probiotic bacteria.

Raw milk, obtained from a near dairy factory (Milkaut Coop. Ltda., Franck, Santa Fe, Argentina), was batch pasteurized at 65 °C for 20 min, and cooled to 37 °C. Calcium chloride (Merck, Darmstadt, Germany) was added to a final concentration of 0.02% (w/v). After that, milk was divided in three aliquots of 45 L each. As mentioned above, our pilot plant has only two vats for simultaneous work so we manufactured two cheeses first, and the third after they were molded. Fat concentration was standardized at 3.8% (w/v) for control and EL cheeses and at 3.49% (w/v) for EP cheese, because in the latter the addition of the substrate increased the fat concentration to a final content of 3.8% (w/v). Besides, the same amount of milk powder used in the preparation of the substrate was added to cheese-making milk for C and EL cheeses, in order to obtain the same non fat solid content in all cheeses. *Streptococcus thermophilus* (Diagramma, Santa Fe, Argentina) was used as primary starter. The lyophilized culture of *S. thermophilus* was dispersed in a small quantity of pasteurized milk, maintained for 5–10 min at 37 °C and then added to cheese-making milk to obtain a concentration of 10^6 CFU mL^{-1}. For EL cheese the lyophilized probiotic culture was mixed with the primary starter, and both were dispersed in milk and added to the vat as described before. For EP cheese, the lyophilized probiotic culture was pre-incubated in a substrate described below, and added to cheese-making milk after the primary starter. In both types of experimental cheeses, probiotic bacteria were added at a concentration sufficient to achieve 10^6 CFU mL^{-1} of cheese-making milk. After 15 min, 1 g of chymosin produced by fermentation of genetically modified *Kluyveromyces lactis* (Maxiren 150, Gist Brocades, France) was dispersed in 25 mL of distilled water and added to the vat. When the curd reached the appropriate strength, it was cut in successive steps (with manual stirring between steps) until it was the size of a corn grain (at 37 °C – approx. 20 min). The mixture of curd particles and whey was gently stirred and heated at the rate of 1 °C min^{-1} until 45 °C for 15–20 min approximately, in order to reduce the humidity of curd grains.

After that, the curd was separated from whey and molded. The three moulds were piled and pressed during 24 h (0.2–0.3 kg cm^{-2}). Young cheeses were brined in 20% (w/v), pH 5.40, brine during 24 h and ripened for two months at 12 °C and 80% relative humidity. According to Argentinean legislation, Pategrás cheese must be ripened at least for 45 days, but we prolonged this period until 60 days, in order to establish if probiotic bacteria remained viable also after the minimal ripening period, and therefore the product could have a more extended shelf life.

2.2. Probiotic cultures

Lyophilized commercial cultures of two *Lactobacillus* species were used. In trial 1, a strain of *L. acidophilus* was studied, whereas in trial 2 a strain of *Lactobacillus paracasei* subsp. *paracasei* was tested.

The substrate used for addition of probiotic bacteria to EP cheeses was prepared according to a modified method of Daigle, Roy, Bélanger, and Vuillermard (1999). Water was added to a mixture of skim milk powder (Molico, Nestlé Argentina, Buenos Aires, Argentina) and raw, freshly obtained cream, 40% fat (w/w) (Milkaut Coop. Ltda., Franck, Argentina) to reach a final concentration of 14% (w/v) fat and 5.2% (w/v) proteins. The substrate was treated at 80 °C during 5 min and then cooled at 37 °C. Probiotic culture was added to this substrate to attain approximately 5×10^7 CFU mL^{-1}. The inoculated substrate was incubated at 37 °C – 5 h and then stored at 4 °C until the next day, when it was used to make EP cheese.

2.3. Selection of culture media

Different culture media were tested for the differential plate counts of probiotic and lactic acid starter.

L. acidophilus and *L. paracasei* subsp. *paracasei* were tested in Skim Milk Agar (SMA) (Frank, Christen, & Bullerman, 1993), MRS (Britania, Buenos Aires, Argentina) agar and Bile-MRS agar (Vinderola & Reinheimer, 1999). *L. acidophilus* was also tested in acidified MRS agar (International Dairy Federation, 1988). *S. thermophilus* count was performed on Skim Milk Agar (SMA).

2.4. Fat substrate analysis

During the incubation and cold storage of the substrate, pH values and probiotic bacteria plate counts were determined at 0, 2, 5 and 20 h.

In order to assess the number of probiotic bacteria, decimal dilutions of the sample were made in 0.1% casein peptone (Microquim, Santa Fe, Argentina) water,

and 0.1 mL aliquots of the appropriate dilutions were plated on MRS agar. Plates were incubated in aerobic conditions at 37 °C for 48 h.

2.5. Gross composition of the cheeses

Gross composition was assessed on 3-day-old cheeses. Dry extract was analyzed by drying the sample at 105 °C until constant weight according to IDF standards (International Dairy Federation, 1982). Fat matter (by Gerber's method) and pH were determined according to standard of American Public Health Association (APHA) (Bradley et al., 1993). Protein content was determined by the Kjeldahl method according to IDF standards (International Dairy Federation, 1993). Sodium chloride content was analyzed after 30 days of ripening to allow a better distribution of the salt in the cheese, following a standard spectrophotometric method (AOAC, 1990).

2.6. Microbiologic analysis of cheese and whey

Lactobacilli were enumerated in whey drained just before curd molding. Decimal dilutions in 0.1% casein peptone water were made and aliquots of the appropriate dilutions were plated in MRS agar and incubated as described above.

Populations of lactic starter and probiotic adjunct culture were enumerated in cheese on aseptic samples taken at 0, 3, 15, 30, 45, and 60 days of ripening. Ten grams of cheese was emulsified with 90 mL of sterile sodium citrate (2%, p/v) in a Stomacher 400C lab blender (Brinkmann, NY, USA). Decimal dilutions in 0.1% casein peptone water were made. Aliquots of the appropriate dilutions were plated in SMA for primary starter count and in MRS agar for probiotic culture count. Plates were incubated in aerobic conditions at 37 °C during 48 h.

2.7. Sensory analysis

An informal sensory analysis of the cheeses was performed by a non trained panel and was aimed to detect differences between cheeses with and without the addition of probiotic lactobacilli. The panelists were asked to classify samples as "good", "fair" or "disagreeable", based on overall quality, and allowed to describe defective characteristics if there were any.

2.8. Statistical analysis

Data were processed by one way ANOVA with Statistix 7 (Analytical Software, Tallahassee, USA). When differences were found, means were compared by the least significant difference test (LSD) using the same tool.

3. Results and discussion

3.1. Selection of culture media

Culture media were selected in order to obtain satisfactory cell recovery and reliable morphologic differentiation between probiotic bacteria and primary starter. *L. acidophilus* did not grow on acidified MRS agar. Efficiency of cell recovery was similar for *L. acidophilus* and *L. paracasei* subsp. *paracasei* in Bile-MRS agar, MRS agar and SMA. Taking into account these results, MRS agar was chosen for lactobacilli plate count. This medium is recommended for lactobacilli (de Man, Rogosa, & Sharpe, 1960), and its preparation was easier than that of Bile-MRS agar. On the other hand, *S. thermophilus* colonies showed a characteristic morphology on MRS agar, completely different from probiotic bacteria colonies, and therefore they did not interfere in the probiotic enumeration. On MRS agar *L. acidophilus* and *L. paracasei* yielded irregular light grey and round beige creamy colonies, respectively, while colonies of *S. thermophilus* were much smaller, point-shaped and white.

Finally, the probiotic lactobacilli did not interfere in the plate count of *S. thermophilus* on SMA, because colonies were also different in this medium. Lactobacilli yielded similar colonies to those obtained on MRS agar, while colonies of *S. thermophilus* were round and white.

3.2. Evolution of pH and probiotic bacteria viability during the incubation of the substrate

An significant ($p < 0.05$) increase in lactobacilli population was observed during the incubation of the substrate inoculated with *L. acidophilus*, which went along with a significant ($p < 0.05$) decrease in pH values (Table 1). On the other hand, when *L. paracasei* subsp. *paracasei* was inoculated to the substrate, changes in the lactobacilli population and pH were also significant ($p < 0.05$). However, the reduction of pH values was lower than in the first case (Table 1).

The two studied strains of probiotics remained viable throughout the incubation and storage of the substrate, granting an appropriate number of probiotic bacteria to inoculate milk for EP cheese-making.

3.3. Gross composition of the cheeses

Values of pH, fat matter, dry extract, sodium chloride and total protein are presented in Table 2.

Control and experimental cheeses did not show significant differences for fat matter, dry extract, sodium chloride content and total protein, both for *L. acidophilus* and *L. paracasei* subsp. *paracasei* trials.

Cheeses produced with the addition of *L. acidophilus* had a pH value significantly lower ($p < 0.05$) than con-

Table 1
pH values and probiotic cell counts during incubation (37 °C for 5 h) and cold storage (4 °C for 15 h) within a substrate composed of milk and milk fat

Time (h)	Trial 1		Trial 2	
	pH	Cell counts ($\log_{10}$ CFU mL^{-1})	pH	Cell counts ($\log_{10}$ CFU mL^{-1})
0	6.50 ± 0.06[a]	6.97 ± 0.31[a]	6.45 ± 0.08[a]	7.89 ± 0.21[a]
2	6.42 ± 0.08[a]	6.82 ± 0.10[a]	6.30 ± 0.05[b]	8.32 ± 0.16[b]
5	6.35 ± 0.05[b]	7.18 ± 0.23[a]	6.25 ± 0.10[b]	8.21 ± 0.07[a]
20	6.00 ± 0.10[c]	7.88 ± 0.33[b]	6.25 ± 0.08[b]	8.70 ± 0.38[b]

Time: total time (incubation + storage).
Trial 1: substrate with *L. acidophilus*. Trial 2: substrate with *L. paracasei* subsp. *paracasei*.
Means in the same column with different superscript differ ($p < 0.05$).

Table 2
Gross composition of 3-day-old cheeses

	Trial 1*			Trial 2**		
	C1	EL1	EP1	C2	EL2	EP2
pH	5.25 ± 0.05[a]	5.08 ± 0.08[a,b]	4.92 ± 0.16[b]	5.20 ± 0.07	5.15 ± 0.18	5.05 ± 0.10
Fat matter (%)	28.70 ± 2.23[a]	29.03 ± 3.02[a]	29.47 ± 2.24[a]	27.00 ± 1.41	27.97 ± 1.05	28.50 ± 1.32
Total protein (%)	22.01 ± 1.14[a]	22.25 ± 0.49[a]	21.71 ± 0.61[a]	21.63 ± 1.89	21.50 ± 1.08	21.47 ± 0.50
Dry extract (%)	55.71 ± 1.35[a]	54.99 ± 2.41[a]	55.23 ± 0.80[a]	54.57 ± 1.12	54.63 ± 0.36	55.24 ± 1.39
Salt in moisture (%)	3.34 ± 0.46[a]	3.85 ± 0.13[a]	3.61 ± 0.19[a]	3.69 ± 0.17	3.52 ± 0.25	3.24 ± 0.20

Means and standard deviation of three replicate cheeses are reported. Trial 1: cheeses with *L. acidophilus* as adjunct starter. Trial 2: cheeses with *L. paracasei* subsp. *paracasei* as adjunct starter. C1 and C2: control cheeses without probiotic bacteria, in trials 1 and 2, respectively. EL1 and EL2: experimental cheeses with addition of probiotic bacteria as a lyophilized culture, in trials 1 and 2, respectively. EP1 and EP2: experimental cheeses with addition of probiotic bacteria pre-incubated in a substrate, in trials 1 and 2, respectively.
* Means in a row with different superscript differ ($p < 0.05$)
** Values in the same row did not differ.

trol cheeses, especially EP1 cheeses. A test for means comparison showed that pH means for C1 and EP1 cheeses belong to different means groups, while EL1 had an intermediate value. As for trial 2, the pH value of cheeses produced with *L. paracasei* subsp. *paracasei* was also lower than control cheeses pH, also specially in EP2 cheeses. However, no significant differences were detected in this case ($p > 0.05$).

Even if the substrate developed some acidity that directly contributed to the drop in pH of cheese-making milk, this contribution was not significant as could be tested during cheese-making (results not shown). The differences in the pH value of the cheeses found 3 days after cheese-making, suggest that probiotic bacteria pre-cultured in the substrate possessed a higher acidifying activity than the same culture dispersed just before use, because of their metabolic status or their number.

To summarize we can point out that the addition of probiotic bacteria did not significantly affect the overall composition of cheeses, except for pH in trial 1, due to over acidification of cheeses type EP1.

3.4. Microbiological analysis of cheeses

Streptococci and lactobacilli plate counts for control cheeses and experimental cheeses from trials 1 and 2, are shown in Figs. 1 and 2, respectively.

Primary starter was about 10^8 CFU g^{-1} in curd samples before molding. After pressing and brining, the streptococci population was one order higher in all cheeses. This number remained more or less constant during ripening, and no significant differences was detected in primary starter number between control and experimental cheeses. These results are consistent with those of Vinderola et al. (2000) who found that *S. thermophilus* population remained over 10^8 CFU g^{-1} in soft cheeses.

As we mentioned above, probiotic bacteria were dosed up in order to attain a number of 10^6 CFU mL^{-1} in cheese-making milk. During separation of curd and whey, the number of probiotic bacteria in curd was between 7.7×10^6 and 1.1×10^8 CFU mL^{-1}, while in whey the count was lower than 10^5 CFU mL^{-1}. Other experiences in Argentinean cheeses with probiotic bacteria (Vinderola et al., 2000), reported a similar number for probiotic population, but in these experiences cheese milk was concentrated by ultrafiltration and the probiotic bacteria were added after this technological step. In the present work we showed that it is possible to add the probiotic bacteria directly to cheese-making milk, without changes in the standard technology, and at the same time to obtain a high number of probiotics in the curd.

In cheeses from trial 1, the probiotic bacteria count in curd was, in average, 7.7×10^6 and 5.2×10^7 CFU g^{-1}

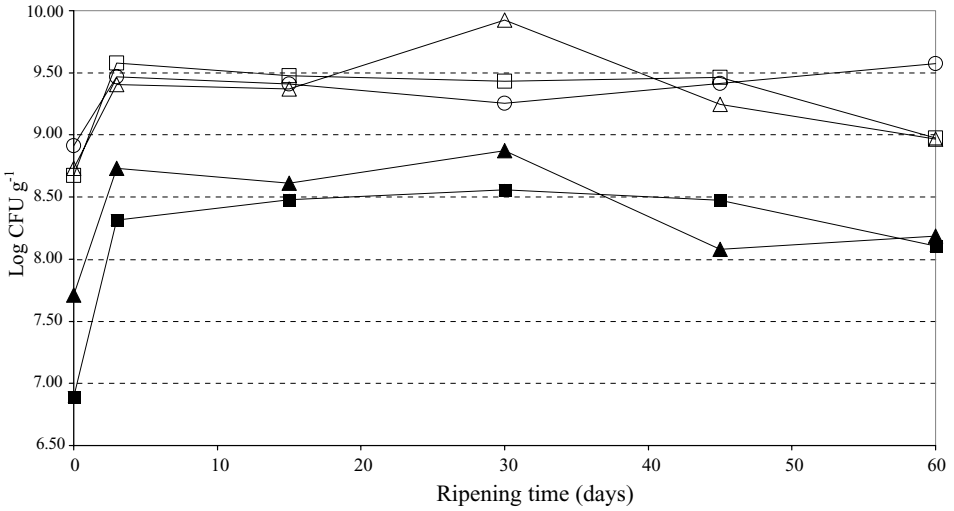

Fig. 1. Evolution of lactic starter and probiotic culture populations during ripening of cheeses from trial 1. (○, □, △) *S. thermophilus* plate count for cheeses C1, EL1 and EP1, respectively. (■, ▲) *L. acidophilus* in cheeses EL1 and EP1, respectively. C1: control cheeses without probiotic bacteria. EL1: experimental cheeses with addition of *L. acidophilus* as a lyophilized culture. EP1: experimental cheeses with addition of *L. acidophilus* pre-incubated in a substrate.

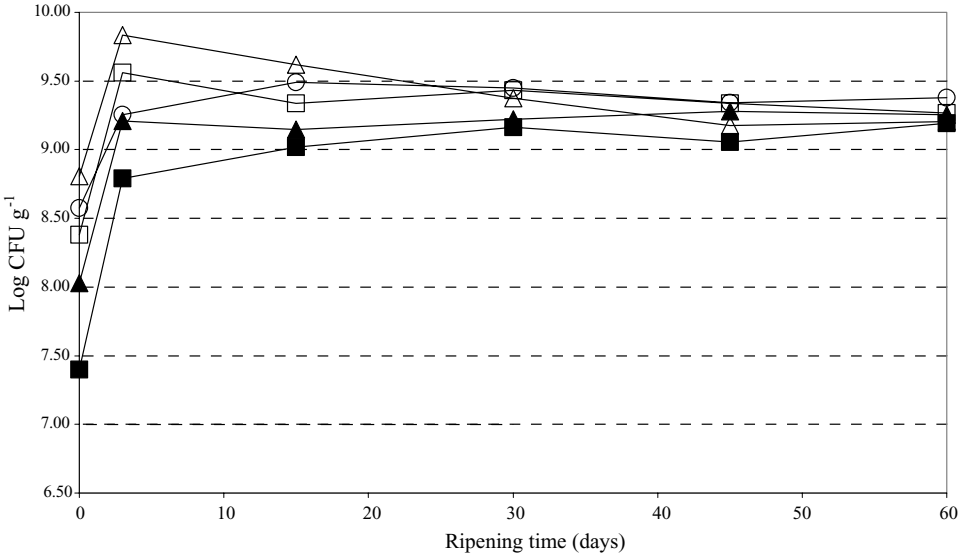

Fig. 2. Evolution of lactic starter and probiotic culture populations during ripening of cheeses from trial 2. (○, □, △) *S. thermophilus* plate count for cheeses C2, EL2 and EP2, respectively. (■, ▲) *L. paracasei* subsp. *paracasei* in cheeses EL2 and EP2, respectively. C2: control cheeses without probiotic bacteria. EL2: experimental cheeses with addition of *L. paracasei* subsp. *paracasei* as a lyophilized culture. EP2: experimental cheeses with addition of *L. paracasei* subsp. *paracasei* pre-incubated in a substrate.

for cheeses EL1 and EP1, respectively. This significant difference ($p < 0.05$) was not surprising because *L. acidophilus* population increased almost in a log cycle during the incubation in the substrate. At three days of ripening the number was about 10^8 CFU g^{-1}, and remained constant until the end of ripening. Lactobacilli count in EP1 cheeses was always higher than in EL1 cheeses, except for the sample obtained at 45 days. However differences were significant only for 0 and 3 days of ripening.

In cheeses from trial 2, the average number of lactobacilli in the curd was 2.5×10^7 and 1.1×10^8 CFU g^{-1} for cheeses EL2 and EP2, respectively. After three days of ripening, plate counts for lactobacilli were 6.2×10^8 and 1.6×10^9 CFU g^{-1}, respectively. From 15 to 60 days of ripening, probiotic bacteria population remained near 10^9 CFU g^{-1} for both EL2 and EP2 cheeses. As in the first trial, probiotic bacteria number was always higher in EP2 cheeses; however, differences were not significant in trial 2.

Inhibition was not detected between lactic and probiotic populations, which is a feature that should be regarded in order to design new probiotic products (Vinderola et al., 2002b). *S. thermophilus* plate count

number did not decrease during ripening, contrarily to certain mesophilic lactic cultures which can die and lyse during the ripening period. This has been suggested as a benefit to adjunct culture survival and growth (Thomas, 1987), however in our cheese model, the non lytic starter was perfectly compatible with probiotic survival.

As a consequence of these results it can be stated that the addition of probiotics after pre-incubation in the substrate did not improve their survival during cheese ripening. The substrate not only did not enhance the protection of probiotic bacteria, but also was a more complex methodology than direct addition of lyophilized culture. First, it was more time consuming, and in the second place, pre-incubation could be a sensitive step taking into account contamination and phage attack (Batt, Erlandson, & Bsat, 1995). Finally, the addition of probiotics within the substrate always provided over acidified cheeses. However, the methodology presented an advantage: pre-incubation in the substrate increased the probiotic population in the inoculum almost in a log cycle, which can contribute to diminish the costs of probiotic cultures for the dairy industry.

We also found that the strain of *L. paracasei* attained and maintained a higher viability in the cheese matrix than *L. acidophilus*, however the cheeses obtained in both trials contained more probiotics than the required dose. Vinderola, Costa, Regenhardt, and Reinheimer (2002a) observed a higher susceptibility of *L. acidophilus* to acid stress, when compared with other lactic and probiotic bacteria, which may explain the slight diminution of *L. acidophilus* population of EP1 cheeses at the end of the ripening.

3.5. Sensory analysis

Experimental cheeses manufactured with the addition of a strain of *L. acidophilus* in the substrate (EP1) were qualified only as "fair" by the panel, while all the other cheeses were described as "good". Panelists found that EP1 cheeses were more crumbly, white and acid than the other samples. These differences are not surprising taking into account the lower pH value of EP1 cheeses.

4. Conclusions

Addition of probiotic bacteria as a lyophilized powder was a more efficient procedure than their addition within a substrate composed of milk and milk fat, because it was easier, the probiotic population at the end of ripening was quite similar, and cheeses were not over acidified. However, further research about the addition methodology within the substrate should not be discarded, because it appears to be advantageous from an economic point of view. A lower initial probiotic population may be inoculated to the substrate to obtain cheeses with a similar or slightly minor probiotic population, and no over acidification.

As the addition of probiotic bacteria to a food product should have a positive, or at least negligible effect on its sensory characteristics, the tested strain of *L. acidophilus* was not as promising as the strain of *L. paracasei* for the manufacture of probiotic Pategrás cheese. However, if it is added directly as a lyophilized culture, it would be equally suitable.

In the present study we found that the tested probiotic strains were viable during ripening at levels of 10^8 CFU g^{-1} in the semi-hard cheeses produced. These levels are higher than the required to meet probiotic food standards.

We conclude that Pategrás Argentino cheese can be an appropriate vehicle for delivering the tested strains of probiotic bacteria through human diet.

Acknowledgements

Authors acknowledge the financial support of Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT01 09-08040 BID 1201 OC/AR).

References

Adhikari, K., Mustapha, A., Grün, I. U., & Fernando, L. (2000). Viability of microencapsulated bifidobacteria in set yogurt during refrigerated storage. *Journal of Dairy Science, 83*(9), 1946–1951.

ANMAT. (1999). *Código Alimentario Argentino*. Administración Nacional de Alimentos, Medicamentos y Tecnología Médica, Ministerio de Salud. De la Canal y Asociados, Buenos Aires, Argentina.

AOAC, Official Methods of analysis. (1990). Minerals in ready-to-feed milk-based infant formula. Atomic absorption spectrophotometric method No. 985.35. Arlington, VA, USA.

Batt, C. A., Erlandson, K., & Bsat, N. (1995). Design and implementation of a strategy to reduce bacteriophage infection of dairy starter cultures. *International Dairy Journal, 5*, 949–962.

Blanchette, L., & Roy, D. (1995). Production of cultured cottage cheese dressing by bifidobacteria. *Journal of Dairy Science, 78*(7), 1421–1429.

Blanchette, L., Roy, D., Bélanger, G., & Gauthier, S. F. (1996). Production of cottage cheese using dressing fermented by bifidobacteria. *Journal of Dairy Science, 79*(1), 8–15.

Bradley, R. L., Arnold, E., Barbano, D. M., Semerad, R. G., Smith, D. E., & Vines, B. K. (1993). Chemical and physical methods. In R. Marshall (Ed.), *Standard methods for the examination of dairy products* (pp. 433–531). Washington, DC: American Public Health Association.

Corbo, M. R., Albenzio, M., De Angelis, M., Sevi, A., & Gobbetti, M. (2001). Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with bifidobacteria. *Journal of Dairy Science, 84*(3), 551–561.

Daigle, A., Roy, D., Bélanger, G., & Vuillermard, J. C. (1999). Production of probiotic cheese (cheedar-like cheese) using enriched cream fermented by *Bifidobacterium infantis*. *Journal of Dairy Science, 82*(6), 1081–1091.

Davidson, R. H., Duncan, S. E., Hackney, C. R., Eigel, W. N., & Boling, J. W. (2000). Probiotic culture survival and implications in fermented frozen yogurt characteristics. *Journal of Dairy Science, 83*(4), 666–673.

de Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*, 130–135.

De Vuyst, L. (2000). Technology aspects related to the application of functional starter cultures. *Food Technology and Biotechnology, 38*(2), 105–112.

Dinakar, P., & Mistry, V. V. (1994). Growth and viability of *Bifidobacterium bifidum* in cheedar cheese. *Journal of Dairy Science, 77*(10), 2854–2864.

Donnet-Hughes, A., Rochat, F., Serrant, P., Aeschlimann, J. M., & Schiffrin, E. J. (1999). Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. *Journal of Dairy Science, 82*(5), 863–869.

Fox, P. F. (2003). Cheese. Overview. In H. Roginski, J. Fuquay, & P. Fox (Eds.), *Encyclopedia of dairy sciences* (pp. 252–261). London: Academic Press.

Frank, J. F., Christen, G. L., & Bullerman, L. B. (1993). Tests for groups of microorganisms. In R. Marshall (Ed.), *Standard methods for the examination of dairy products* (pp. 271–286). Washington, DC: American Public Health Association.

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology, 66*(5), 365–378.

Gardiner, G. E., Bouchier, P., O'Sullivan, E., Kelly, J., Collins, J. K., Fitzgerald, G., et al. (2002). A spray-dried culture for probiotic cheddar cheese manufacture. *International Dairy Journal, 12*, 749–756.

Gardiner, G., Stanton, C., Lynch, P. B., Collins, J. K., Fitzgerald, G., & Ross, R. P. (1999). Evaluation of cheddar cheese as a food carrier for delivery of a probiotic strain to the gastrointestinal tract. *Journal of Dairy Science, 82*(7), 1379–1387.

Gobbetti, M., Corsetti, A., Smacchi, E., Zocchetti, A., & De Angelis, M. (1998). Production of crescenza cheese by incorporation of bifidobacteria. *Journal of Dairy Science, 81*(1), 37–47.

Gomes, A. M. P., Malcata, F. X., Klaver, F. A. M., & Grande, H. J. (1995). Incorporation and survival of *Bifidobacterium* sp. strain Bo and *Lactobacillus acidophilus* strain Ki in a cheese product. *Netherlands Milk and Dairy Journal, 49*, 71–95.

Heller, K. J. (2001). Probiotic bacteria in fermented foods: product characteristics and starter organisms. *American Journal Clinical Nutrition, 73*(suppl), 374S–379S.

http://www.cil.org.ar.

International Dairy Federation. (1982). Formaggio e formaggio fuso. Determinazione della materia secca. Metodo di riferimento. No. 4A. IDF, Brussels, Belgium.

International Dairy Federation. (1993). Latte. Determinazione del tenore in azoto. Metodo di riferimento, No. 20(B). IDF, Brussels, Belgium.

International Dairy Federation. (1988). Yogurt. Enumeration of characteristic microorganisms. Colony count technique at 37 °C. Bulletin No. 117A/1988. IDF, Brussels, Belgium.

Lynch, C. M., Muir, D. D., Banks, J. M., McSweeney, P. L. H., & Fox, P. F. (1999). Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on cheddar cheese ripening. *Journal of Dairy Science, 82*(8), 1618–1628.

O'Riordan, K., & Fitzgerald, G. F. (1998). Evaluation of bifidobacteria for the production of antimicrobial compounds and assessment of performance in cottage cheese at refrigeration temperature. *Journal of Applied Microbiology, 85*, 103–114.

Rogelj, I., Bogovič Matijašić, B., Majhenič, A. C., & Stojković, S. (2002). The survival and persistence of *Lactobacillus acidophilus* LF221 in different ecosystems. *International Journal of Food Microbiology, 76*, 83–91.

Ross, R. P., Fitzgerald, G., Collins, K., & Stanton, C. (2002). Cheese delivering biocultures – probiotic cheese. *The Australian Journal of Dairy Technology, 57*(2), 71–78.

Roy, D., Mainville, I., & Mondou, F. (1997). Selective enumeration and survival of bifidobacteria in fresh cheese. *International Dairy Journal, 7*, 785–793.

Sanders, M. E. (1998). Overview of functional foods: emphasis on probiotic bacteria. *International Dairy Journal, 8*, 341–347.

Stanton, C., Gardiner, G., Lynch, P. B., Collins, J. K., Fitzgerald, G., & Ross, R. P. (1998). Probiotic cheese. *International Dairy Journal, 8*, 491–496.

Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P., & Kailasapathy, K. (2000). Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. *International Journal of Food Microbiology, 62*, 47–55.

Sun, W., & Griffiths, M. W. (2000). Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan-xanthan beads. *International Journal of Food Microbiology, 61*(1), 17–25.

Thomas, T. (1987). Cannibalism among bacteria found in cheese. *New Zealand Journal Dairy Science Technology, 22*, 215–219.

Vinderola, C. G., Costa, G. A., Regenhardt, S., & Reinheimer, J. A. (2002a). Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *International Dairy Journal, 12*, 579–589.

Vinderola, C. G., Mocchiutti, P., & Reinheimer, J. A. (2002b). Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products. *Journal of Dairy Science, 85*(4), 721–729.

Vinderola, C. G., Prosello, W., Ghiberto, D., & Reinheimer, J. A. (2000). Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in argentinian fresco cheese. *Journal of Dairy Science, 83*(9), 1905–1911.

Vinderola, C. G., & Reinheimer, J. A. (1999). Culture media for the enumeration of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in the presence of yoghurt bacteria. *International Dairy Journal, 9*, 497–505.

Vinderola, C. G., & Reinheimer, J. A. (2000). Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria in fermented dairy products. *International Dairy Journal, 10*, 271–275.

Zalazar, C., Meinardi, C., & Hynes, E. (1999). *Quesos Típicos Argentinos. Una revisión general sobre producción y características*. Universidad Nacional del Litoral, Santa Fe, Argentina: Centro de Publicaciones.

Ziemer, C. J., & Gibson, G. R. (1998). An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. *International Dairy Journal, 8*, 473–479.