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Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage

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Abstract

The survival of *Bifidobacterium bifidum* BBI and *Lactobacillus acidophilus* LAI in reduced-fat (liquid) and full-fat (set) yoghurts produced with two commercial lactic starter cultures (SID and SISD) was investigated. The viability of the probiotic bacteria was also assayed in milk acidified with lactic acid at different pH values. Samples were stored at 5°C for up to 4 weeks. There was a great variability in the survival ability of the probiotic cultures in the two yoghurt types. *L. acidophilus* LAI demonstrated, in general, a lower resistance to the yoghurt environment than *B. bifidum* BBI. On the other hand, the full-fat yoghurt was a more inhibitory medium than the reduced-fat one, especially for *B. bifidum* BBI. Regarding the lactic starters used, the results showed that the culture SISD was clearly more inhibitory for both probiotic organisms than the culture SID. The loss of cell viability in yoghurt samples was different (higher in some cases and lower in others) from that due to lactic acid only. In general, pH values of 4.5 or lower jeopardised the cell viability of the probiotic organisms in yoghurt stored at 5°C. This work shows the importance of selecting a suitable combination of probiotic strains and starter cultures when different yoghurt types are formulated.

Keywords: Bacterial survival; Probiotics; Yoghurt; Storage

1. Introduction

In recent years, there has been a worldwide increasing interest in the addition of intestinal bacterial species (*Bifidobacterium* spp. and *Lactobacillus acidophilus*/*casei*) to fermented milks. The addition of probiotic bacteria is made not only because of certain claimed health-promoting effects in the intestinal tract (Klaver, Kingma & Weerkamp, 1993) but also because of the sensory aspects as well as the expanding variety of products that can be formulated with them (Kneifel, Jaros & Erhard, 1993). The main "probiotic effects" attributed to these bacteria are: enhancement of immunity against intestinal infections and improvement in lactose utilisation, prevention of diarrhoeal diseases, colon cancer, hypercholesterolaemia and upper gastrointestinal tract diseases, stabilisation of the gut mucosal barrier (Kailasapathy & Rybka, 1997), formation or reconstruction of a well-balanced indigenous intestinal microflora, improvement of calcium absorption and vitamin synthesis and pre-digestion of proteins (Nakasawa & Hosono, 1992; Wood, 1992).

To perform their probiotic action these bacteria must arrive at the intestinal tract alive. This requires their survival in the food used as a vehicle during its shelf-life and after consumption, and their resistance to the acidic conditions of the stomach as well as to bile salts in the small intestine (Kailasapathy & Rybka, 1997). Taking into account all these barriers, it is regarded as essential that: (a) the carrier food contains at least 10^6 viable cells of the probiotic microorganism per gram, (b) the species are of human origin (*L. acidophilus*, *L. casei*, *B. bifidum*, *B. longum*, *B. adolescentis*, or *B. infantis*), and (c) the total intake per week of the product is approximately 300–400 g (Samona & Robinson, 1994).

Some studies have been carried out with the objective of monitoring the survival of the constitutive microflora and the intestinal probiotic bacteria added to the different fermented milks (Beerens, 1990; International Dairy Federation [IDF], 1995; Lapierre, Underland & Cox, 1992; Lim, Huh, & Baek, 1995; Martin & Chou, 1992; Nu-trish Cultures Catalog, 1996; Pacher & Kneifel, 1996; Reinheimer & Vinderola, 1998; Rybka & Kailasapathy, 1996; Silvi, Rumney & Rowland, 1996; Dave & Shah, 1996;).

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In Argentina, there is still a tendency to produce yoghurts with a relatively high acidity level (pH values ranging from 4.0 to 4.5). Lactic acid starters are used with direct (DVS) and semidirect inoculation. On the other hand, a minimal content (10^6 CFU g^{-1}) was established for bifidobacteria added to fermented milks by regulations recently approved (Pagano, 1998) by the countries of MERCOSUR (Argentina, Paraguay, Brazil and Uruguay). Regulatory levels for *L. acidophilus* in these products have not yet been established. However, the high acidity of Argentinian fermented milks generates doubts about the viability of the probiotic microflora.

The aim of this work was to evaluate the survival of lactic acid and intestinal probiotic bacteria in Argentinian commercial yoghurts during refrigerated storage, as well as the effect of the lactic acid content on cell viability.

2. Materials and methods

2.1. Starters and probiotic cultures

Two commercial lactic acid starter cultures (lyophilised form) were used for reduced-fat (liquid) and full-fat (set) yoghurts. Direct inoculation was done with SID (Centro Sperimentale del Latte, Italy) and indirect inoculation was performed with SISD (Centro Sperimentale del Latte, Italy). Both cultures contained *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The lyophilised probiotic cultures used were *B. bifidum* BBI and *L. acidophilus* LAI, from our collection. These cultures are widely used in Argentinian fermented milks.

2.2. Samples

Industrial productions of both, liquid reduced-fat (fat 0.2% w/w, proteins 3.7% w/w and carbohydrates 5.2% w/w) and set full-fat (fat 3.0% w/w, proteins 3.0% w/w and carbohydrates 16.5% w/w) yoghurts were performed in a local dairy plant. Both yoghurt types were produced using the lactic cultures SID and SISD. For each condition (liquid yoghurt/culture SID, liquid yoghurt/culture SISD, set yoghurt/culture SID and set yoghurt/culture SISD), three productions were carried out. The lactic and probiotic cultures were inoculated at the beginning of batch fermentations. All the cultures were used according the manufacture's instructions. Samples from the batch fermentations were taken to the laboratory and stored at 5°C for up to 4 weeks. Cell counts and pH measurements were performed weekly.

2.3. Resistance of probiotic cultures to lactic acid

To study the influence of the lactic acid on the survival of *B. bifidum* and *L. acidophilus*, each bacterium (as a lyophilised culture) was added to acidified milk. Reconstituted (10%) and sterilised (30 min, 110°C) skim milk (RSM) was acidified with lactic acid (Anedra, Buenos Aires, Argentine) to pH 5.5; 4.5 and 3.5. RSM without the addition of lactic acid was used as a control. The acidified and the control milks were inoculated with the probiotic cultures at a concentration of 10^7–10^8 CFU ml^{-1}, distributed in 10 ml sterile flasks and maintained at 5°C for 4 weeks. Counts of viable cells were performed weekly.

2.4. Counts

Samples of 1 ml of yoghurt or acidified milk were decimally diluted in sterile peptone water (0.1%) and 0.1 ml aliquot dilutions plated over the culture media. Skim milk agar (SMA) (Marshall, 1992) was used for viable counts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The selective enumeration of *L. acidophilus* was performed on MRS-bile agar (IDF, 1995). Both media were incubated aerobically at 37°C for 72 h. To obtain *B. bifidum* counts, MRS-LP agar (Vinderola & Reinheimer, 1999) was used, incubated anaerobically (GasPak System-OXOID) during 72 h at 37°C. In a previous work (Vinderola & Reinheimer, 1999) it was demonstrated that MRS-bile agar and MRS-LP agar are able to inhibit the growth of lactic acid bacteria from the starter culture. Besides, *L. acidophilus* growth is inhibited on MRS-LP agar and an aerobic incubation on MRS-bile prevents the development of *Bifidobacterium bifidum*. To confirm the identity of the colonies, cell morphology (phase contrast, 1000x Microscope Jenamed 2 CARL ZEISS) was observed.

MRS agar (De Man, Rogosa & Sharpe, 1960) was used for the enumeration of pure cultures of both *L. acidophilus* and *B. bifidum*. The incubation conditions for *L. acidophilus* and *Bifidobacterium bifidum* enumeration were aerobic and anaerobic (37°C for 72 h), respectively.

2.5. pH

pH measurements were carried out weekly by means of a digital pH-meter (ORION model SA 720).

3. Results

3.1. Changes in microflora and pH in yoghurt

Figs. 1 and 2 show the viability of the lactic starter- and probiotic bacteria in reduced-fat and full-fat yoghurts, respectively, as well as the changes in pH, during the refrigerated storage. Yoghurts produced with the starter SID (direct inoculation) contained a lactic acid bacteria concentration higher than 10^7 CFU ml^{-1}.

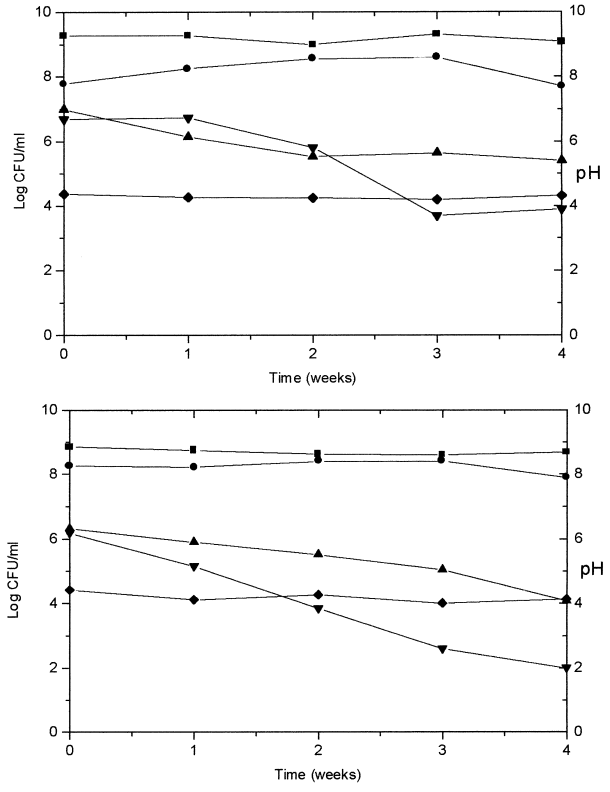

Fig. 1. Changes in pH values (◆) and viable cells counts of *S. thermophilus* (■), *L. delbrueckii* subsp. *bulgaricus* (●), *L. acidophilus* (▲) and *B. bifidum* (▼) in reduced-fat yoghurt, manufactured with the lactic starter SID (top) and SISD (bottom), at 5°C (the values are the mean of three determinations).

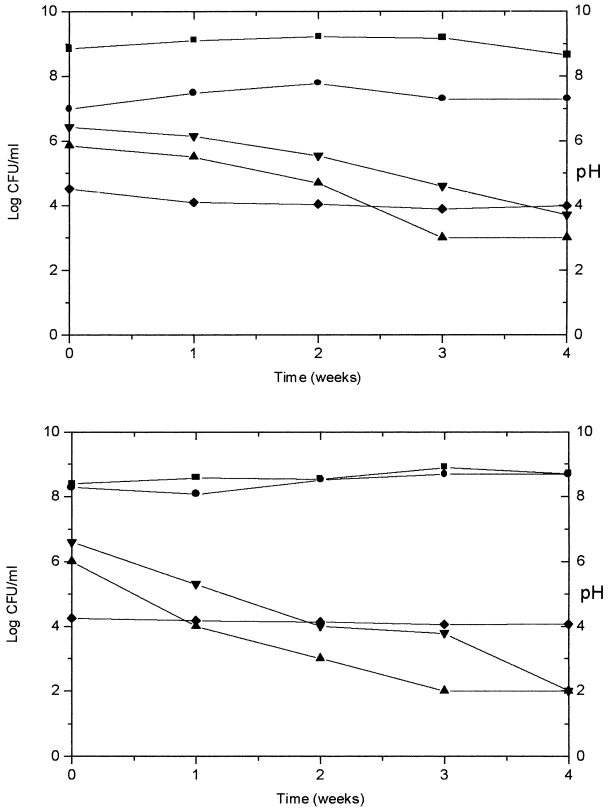

Fig. 2. Changes in pH values (◆) and viable cells counts of *S. thermophilus* (■), *L. delbrueckii* subsp. *bulgaricus* (●), *L. acidophilus* (▲) and *B. bifidum* (▼) in full-fat yoghurt, manufactured with the lactic starter SID (top) and SISD (bottom), at 5°C (the values are the mean of three determinations).

S. thermophilus counts were higher —by at least 1 log order — than those for *L. delbrueckii* subsp. *bulgaricus* . In yoghurts produced with the lactic culture SISD (semi-direct inoculation), the initial contents of both lactic acid bacteria were similar (approximately 10^8 to 10^9 CFU ml^{-1}). In every case, at the end of the storage, the counts of starter bacteria were not significantly different ($P > 0.05$) from the initial ones.

Regarding the probiotic microflora, the results obtained showed that its counts decreased during storage. The rate of this loss in cell viability depended on the yoghurt type and the lactic starter used. Initial counts of *L. acidophilus* LAI and *B. bifidum* BBI ranged from 10^6 to 10^7 CFU ml^{-1}, while the final counts were lower than 10^4 CFU ml^{-1} (except *L. acidophilus* in reduced-fat yoghurt produced with the starter SID). Table 1 shows the decrease of probiotic microflora cell viability in the different yoghurt types. In general, larger reductions were found in full-fat yoghurts. For *B. bifidum* BBI the viability loss values ranged from 1.6 to 4.0 log orders, being higher when lactic starter SISD was used (2.2 and 4.0 log orders in fat-reduced and full-fat yoghurts, respectively). *L. acidophilus* LAI was the most sensitive probiotic bacterium, since higher values of cell viability decrease were found for it (ranging from 2.7 to 4.6 log orders). In this case, the use of the lactic acid starter SISD was also clearly more inhibitory for its cell viability.

The initial pH value for the different yoghurt types ranged from 4.30 to 4.52 (Figs. 1 and 2). In reduced-fat (lactic starter SID) and full-fat (lactic starter SISD) yoghurts, the changes in pH values during the storage were negligible. Instead, in the other two cases, pH values decreased approximately 0.5 units.

3.2. Changes in the probiotic microflora in lactic acid acidified milk

Fig. 3 shows the changes in cell counts of *B. bifidum* BBI and *L. acidophilus* LAI in milk acidified with lactic acid at different pH values. For *B. bifidum* BBI there were no significant differences ($P > 0.05$) in the viable cell counts found at the end of storage of milk acidified to pH 6.5, 5.5 and 4.5. In these cases, the death kinetics was identical and the diminution in cell counts was 2.5 log orders. At lower pH values (3.5), the decrease in cell counts was significantly higher (2.5 log orders after 1 week). On the other hand, *L. acidophilus* LAI was more

Table 1
Diminution in viable cells counts (log orders) of *B. bifidum* BBI and *L. acidophilus* LAI after 4 weeks at 5°C for different kinds of yoghurts and starter cultures used (the values are the mean of three determinations)

Probiotic culture	Fat-reduced (liquid) yoghurt		Full-fat (set) yoghurt	
	Starter culture used			
	SID	SISD	SID	SISD
B. bifidum BBI	1.6 ± 0.26	2.2 ± 0.15	2.9 ± 0.21	4.0 ± 0.24
L. acidophilus LAI	2.8 ± 0.23	4.2 ± 0.19	2.7 ± 0.17	4.6 ± 0.22

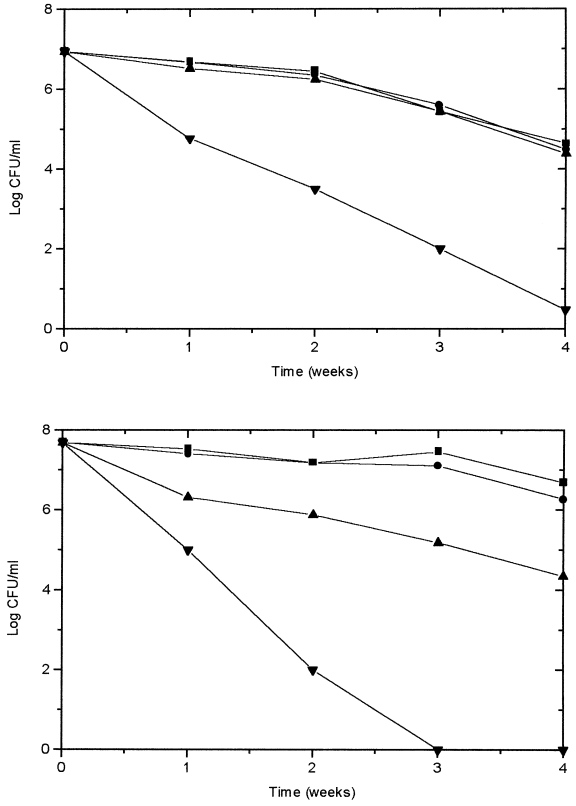

Fig. 3. Survival of *B. bifidum* BBI (top) and *L. acidophilus* LAI (bottom) during refrigerated storage at 5°C in milk acidified with lactic acid at pH 6.5 (■), 5.5 (●), 4.5 (▲) and 3.5 (▼) (the values are the mean of three determinations).

resistant than *B. bifidum* BBI to lactic acid at pH 6.5 and 5.5 since the diminution in cell counts at both pH values was of 1 log order approximately. However, at lower pH values *L. acidophilus* LAI was more inhibited by lactic acid than *B. bifidum* BBI since the fall in cell counts was 3.5 log orders after 4 weeks (pH 4.5) and 9 days (pH 3.5), respectively.

The initial pH values did not change significantly ($P > 0.05$) during the refrigerated storage (data not shown).

4. Discussion

The addition of probiotic bacteria (*Bifidobacterium*, *L. acidophilus* and *L. casei*) to fermented milks is a practice widely adopted by dairy industries. However, it is recognised that there are some physicochemical factors that might condition the survival of probiotic microflora in fermented dairy products, the most important being: yoghurt acidity, dissolved oxygen, species interaction, inoculation practice and storage conditions (Kailasapathy & Rybka, 1997).

In Argentina, the production of yoghurts enriched with intestinal probiotic bacteria began several years ago. Nowadays, there is a great variety of these modified yoghurts. However, there are not sufficient studies about the survival ability of the probiotic cultures used during the refrigerated storage. The results of this work showed a great variability in the survival ability of *B. bifidum* BBI and *L. acidophilus* LAI cultures in the different yoghurt types used. *L. acidophilus* LAI demonstrated, in general, a higher sensitivity to the yoghurt environmental characteristics than *B. bifidum* BBI. On the other hand, the full-fat yoghurt was a medium more inhibitory than the fat-reduced one, especially for *B. bifidum* BBI. Regarding the starter cultures used, the results showed that the culture SISD was clearly more inhibitory for both probiotic cultures.

These facts imply that there was a close relationship among the survival of a particular strain of probiotic bacteria, the starter culture used for the fermentation and the characteristics of the product. Similar results were reported previously for yoghurt-related products fermented with commercial starter cultures (Kneifel et al., 1993) and Australian yoghurts (Micanel, Haynes & Playne, 1997). One important and critical fact was that at the expiration date (4 weeks), the contents of *L. acidophilus* and *B. bifidum* were lower than the suggested levels (10^6 CFU g^{-1}) in all the products analysed. These results indicate that these types of yoghurts would not be a suitable vehicle for probiotic bacteria. A similar observation was previously reported for *L. acidophilus* by Gilliland and Speck (1977) and for bifidobacteria by Modler and Villa-García (1993). Nevertheless, Kailasapathy and Rybka (1997) considered the yoghurt as a suitable vehicle for *L. acidophilus* and *B. bifidum*.

This study also demonstrated that yoghurts with a high fat content seems to be more inhibitory for probiotic cultures than other yoghurt types, in contrast to reports by Micanel et al.(1997).

Although the highest decrease in pH was detected in fat-reduced yoghurt (starter SISD) and full-fat yoghurt (starter SID), the highest reduction in viable cell counts was found in full-fat yoghurt (starter SISD) for each probiotic organism. This might imply that the death of probiotic bacteria is not only governed by the acidity of the medium but also by others factors that should be further studied.

L. acidophilus LAI was more sensitive than *B. bifidum* BBI to the acidic media assayed (yoghurts and milk acidified with lactic acid) in the pH range from 3.5 to 4.5. The suitability of bifidobacteria and intestinal lactobacilli in fermented milks reported in the literature is very variable. A poor survival for bifidobacteria in yoghurts was reported by Klaver et al. (1993), Modler and Villa-García (1993) and Rybka and Kailasapathy (1995) but, in contrast, a satisfactory viability was demonstrated by Smaczny and Reinartz (1982), Medina and Jordano (1994) and Samona and Robinson (1994). On the other hand, Gilliland and Speck (1977) reported that yoghurt should not be considered a desirable vehicle for suspending *L. acidophilus*. However, Nighswonger, Brashears and Gilliland (1996) concluded that the ability of intestinal lactobacilli to survive in fermented dairy products (*L. acidophilus* and *L. casei*) was strain dependant, and Rybka and Kailasapathy (1995) demonstrated that *L. acidophilus* could survive in yoghurt at sufficient levels ($>10^6$ CFU ml^{-1}) for up to 26 days.

Our results showed that at pH 4.5, a pH value near to that measured in the industrial yoghurts tested in this study, the reduction in viable cell counts for the probiotic bacteria recorded in the experiments using acidified milk was, in general, different from that recorded in yoghurts. For *B. bifidum* BBI, a decrease in cell viability similar to that determined in milk acidified with lactic acid (2.5 log orders) was only achieved in fat-reduced (starter SISD) and full-fat (starter SID) yoghurts. Regarding *L. acidophilus* LAI, the reductions in cell counts in every yoghurt type were different to that registered in milk acidified with lactic acid (3.3 log orders). For both probiotic organisms, the loss of cell viability in yoghurt samples was higher in some cases and lower in others than those reported in the experiments with acidified milk. This fact indicates that in different Argentinian yoghurt types there are other psycochemical factors that could reduce or increase the inhibitory power of lactic acid against probiotic bacteria. At present, identification studies on these factors are being carried out in our laboratory (compatibility between strains and lactic acid, lactose, sucrose, sweetener, diacetyl, acetoin and acetaldehyde contents).

This work showed the importance of selecting a suitable combination of probiotic bacteria and starter cultures when different yoghurt types are microbiologically formulated. The acidity level of the product as the only parameter is not sufficient for predicting the survival ability of probiotic bacteria.

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