Viability of Probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and Nonprobiotic Microflora in Argentinian Fresco Cheese

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ABSTRACT

We evaluated the suitability of Argentinian Fresco cheese as a food carrier of probiotic cultures. We used cultures of *Bifidobacterium bifidum* (two strains), *Bifidobacterium longum* (two strains), *Bifidobacterium* sp. (one strain), *Lactobacillus acidophilus* (two strains), and *Lactobacillus casei* (two strains) in different combinations, as probiotic adjuncts. Probiotic, lactic starter (*Lactococcus lactis* and *Streptococcus thermophilus*), and contaminant (coliforms, yeasts, and molds) organisms were counted at 0, 30, and 60 d of refrigerated storage. Furthermore, the acid resistance of probiotic and starter bacteria was determined from hydrochloric solutions (pH 2 and 3) of Fresco cheese. The results showed that nine different combinations of bifidobacteria and *L. acidophilus* had a satisfactory viability (count decreases in 60 d <1 log order) in the cheese. Both combinations of bifidobacteria and *L. casei* cultures assayed also showed a satisfactory survival (counts decreased <1 log order for bifidobacteria but no decrease was detected for *L. casei*). On the other hand, the three combinations of bifidobacteria, *L. acidophilus*, and *L. casei* tested adapted well to the Fresco cheese environment. When a cheese homogenate at pH 3 was used to partially simulate the acidic conditions in the stomach, the probiotic cultures had an excellent ability to remain viable up to 3 h. At pH 2, the cell viability was more affected; *B. bifidum* was the most resistant organism. This study showed that the Argentinian Fresco cheese could be used as an adequate carrier of probiotic bacteria.

(**Key words:** *Bifidobacterium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, probiotic cheese, Fresco cheese)

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INTRODUCTION

Dairy products containing probiotic cultures such as bifidobacteria, *Lactobacillus acidophilus*, and *Lactobacillus casei*—selected because of their health-promoting properties—have been produced for many years. The health and nutritional benefits ascribed to these probiotic bacteria include the alleviation of lactose intolerance (23, 27), inhibition of pathogenic microorganisms and viruses, production of vitamins (25), reduction of cholesterol levels (33), tumor inhibitory effects (24), improvement of the immune response (19), stabilization of the gut mucosal barrier, and prevention of diarrhea (15). To perform their claimed benefits, these bacteria must be viable and present in a high number at the time of consumption (10). Furthermore, they must be alive in the final part of the gastrointestinal tract. For this reason, a dairy product should contain at least 10^6 cfu/ml of probiotic bacteria at the time of consumption (1) and should be consumed regularly; the consumption must be higher than 100 g/d (5).

The development of dairy products containing probiotic bacteria (bifidobacteria and intestinal lactobacilli) is, currently, an extremely important topic with industrial and commercial consequences. Incorporation of bifidobacteria into these kinds of products can be difficult because they require anaerobic atmospheres and pH values of 6.5 to 7.0 (7, 10). Several attempts have been made to use dairy products as carriers of bifidobacteria, such as traditional yogurt (26), low-acidity yogurts (22), fermented milks (20), ice cream (11), cultured buttermilk, and powder preparations (5). More recently, a small number of researchers and industries have started to produce cheeses with probiotic bacteria as adjuncts. Most of the work on probiotic cheese production has been carried out with bifidobacteria alone, or mixed cultures of bifidobacteria and *L. acidophilus*, but *L. casei* has rarely been used (8, 31). Bifidobacteria have been used as the sole probiotic adjunct to produce cottage (1, 2, 25), Crescenza (7), Cheddar (4, 5, 29) and white brined (6) cheeses. In the first of them, the survival ability was moderate for *Bifidobacterium infantis* (1, 2) and clearly strain dependent for 20 strains that belonged to seven *Bifidobacterium* species (25). In Cheddar cheese, *B. infantis* (4) and *Bifidobacterium bifidum* (5) remained viable for 12 and 24 wk, respectively. The adaptability of *B. bifidum* and *B. longum* to the Crescenza cheese environment was better than that of *B. infantis* (7). On the other hand, *B. bifidum* and *Bifidobacterium adolescentis* had acceptable and poor survivals, respectively, in white brined cheese (6).

The use of bifidobacteria and *L. acidophilus* as a probiotic mixture adjunct and as starter was reported for semi-hard goat (9) and Gouda (10) cheeses, respectively. In both of them, *Bifidobacterium* and *L. acidophilus* showed satisfactory viability (>10^6 cfu/g) during at least 9 wk. Although the results obtained were quite promising, the success of the addition of probiotic bacteria was very dependent on the species and strains used, metabolic interactions with lactic acid starters, fermentation conditions, pH of the product, presence of oxygen, and storage temperature (2, 7, 24, 28).

Ingested microorganisms are exposed to several stress factors that influence their viability through the gastrointestinal tract (18). Some characteristics to consider when a probiotic strain is added to a fermented dairy product are its ability to survive the acidic conditions of the stomach and the bile acid concentrations commonly encountered in the gastrointestinal tract of humans (3). Incorporation of these microorganisms into cheese appears to be an encouraging alternative to the problem of the survival until consumption because of high pH values of this kind of product, the closed matrix, and the high fat content, which might offer additional protection to these organisms during their passage through the gastrointestinal tract (31).

In Argentina, approximately 37% of total milk production is used in the manufacture of cheeses, of which soft cheeses represent 48.8% (34). This leads to almost 180,000 tonnes/yr of soft cheeses that are absorbed entirely by the internal market. The Fresco cheese is a typical Argentinian soft cheese that appears to be suitable as a carrier of probiotic bacteria, since its relatively high pH value and enriched protein content as a result of ultrafiltration of the milk might be protective factors towards probiotic and nonprobiotic microflora.

The aim of this study was to evaluate the viability of bifidobacteria, *L. acidophilus*, and *L. casei*, in different combinations, during refrigerated storage of Fresco cheese, and their ability to survive in hydrochloric solutions, partially simulating stomach acidity. To the best of our knowledge, this work is the first of its kind carried out in Latin America.

MATERIALS AND METHODS

Cultures

Frozen cultures of bifidobacteria (*B. longum*, strains B1 and B2, *B. bifidum*, strains B3 and B4 and *Bifidobacterium* sp. strain B5), *L. acidophilus* (strains A1 and A2), *L. casei* (strains C1 and C2), *Lactococcus lactis* (strain A6) and *Streptococcus thermophilus* (strain A4) were obtained from local providers. Cultures A4 and A6 were used as lactic starters for Fresco cheese productions. For the experiments of survival through hydrochloric conditions, *B. bifidum* B4, *L. acidophilus* A2, and *L. casei* C1 were used.

Cheese Making and Cheese Samples

Fresco cheese was manufactured at a commercial cheese manufacturing factory near Santa Fe. Lots of 9000 L of raw milk were standardized at the dairy plant to a fat content of 1.8% and stored at 5°C until being processed (maximum 4 h). Then, it was pasteurized following a high temperature—short time process (18 s at 74°C) and cooled. Frozen cultures of lactic and probiotic bacteria were inoculated (1% wt/wt) at the beginning of batch fermentations. All the cultures were used according to the manufacturer's instructions. The inoculated milk was ultrafiltered to a concentration of 40% of total solids and the retentate was maturated (3 h at 42°C). The curd was salted, cut, and vacuum-packed. Finally, the cheese was ripened at 5°C for 12 d before commercial distribution.

For Fresco cheese, the general values of some physicochemical parameters are: pH 5.29 ± 0.11, moisture 58 ± 1.15% wt/wt, fat 12 ± 0.43% wt/wt, proteins 23 ± 0.84% wt/wt, salt 0.9 ± 0.03% wt/wt, ash 3.4 ± 0.05% wt/wt, DM 40.8 ± 1.03% wt/wt, and calcium 0.6 ± 0.04% wt/wt.

For this study, Fresco cheeses with 14 different combinations of probiotic cultures were manufactured in triplicate. The cheeses were grouped in three different categories: BA-cheeses (containing bifidobacteria and *L. acidophilus* as probiotics), BC-cheeses (bifidobacteria and *L. casei*) and BAC-cheeses (bifidobacteria, *L. acidophilus*, and *L. casei*). For BA-cheeses the combinations of probiotic cultures assayed were B1A1, B1A2, B5A1, B5A2, B2A1, B2A2, B3A2, B4A1, and B4A2. For BC-cheeses, the combinations formulated were B5C2 and B4C1. Finally, for BAC-cheeses three combinations were done: B1A1C2, B2A2C2, and B4A2C1. All of them included *S. thermophilus* A4 and *Lactococcus lactis* A6 as acidifying cultures.

Microbiological Analysis

Bacterial counts from cheeses were performed immediately after manufacture, and at 30 and 60 d of cold storage (5°C). Cheese portions (20 g), aseptically sampled, were suspended in 180 ml of 2% sodium citrate solution and homogenized (3 min) in a Stomacher Lab-Blender 400. Serial dilutions were made in sterile peptone water consisting of 0.1% (wt/vol) casein peptone (Microquin, Santa Fe, Argentina) and plated following the surface plate technique on different media for viable counts. Lactic Bacteria Differential agar (Hi-Media, Bombay, India) was used to enumerate *Streptococcus thermophilus* (32), after an incubation of 3 d at 37°C. *Lactococcus lactis* were counted on Elliker agar (Biokar, Beauvais, France) (25°C, 3 d). Bifidobacteria and *L. casei* were enumerated on LP-MRS agar according to Vinderola and Reinheimer (32) and Bile-MRS agar (12) was used to enumerate *Lactobacillus acidophilus* (37°C, 3 d). Only LP-MRS agar was incubated anaerobically (GasPak system, OXOID). The cheeses were also analyzed for coliforms (14), yeasts, and molds (13). All determinations were made in duplicate.

Cell Viability in Hydrochloric Solutions

Homogenates of cheese (see above) were aseptically acidified up to pH 2 and 3 with 1N HCl (wt/vol) (Cicarelli, Argentina). The acidified mixture was distributed in sterile test tubes and incubated at 37°C in a water bath. Viable counts were made at the beginning of the assays and after 30, 60, 120, and 180 min following the enumeration methodology described above. Acidified suspensions of the lactic and probiotic cultures in 2% sodium citrate solutions were used as controls. The experiments were performed in triplicate.

Statistical Analysis

Data from viable counts were analyzed by the one-way ANOVA procedure of SPSS software. The differences among means were detected by Duncan's multiple range test (35).

RESULTS

Tables 1 to 3 show the changes in the probiotic microflora of Fresco cheese during 60 d of storage. The viability of bifidobacteria and *L. acidophilus* in BA-cheeses during storage can be seen in Table 1. These cheeses had counts of bifidobacteria and *L. acidophilus* higher than 10^6 cfu/g to the end of the storage period, except for the B2A1 cheese, where, although no significant differences ($P > 0.05$) were found, colony counts were lower than 10^6 cfu/g at 60 d. The biggest fall in bifidobacteria viability (0.8 log orders approximately after 60 d) was recorded for *B. bifidum* B3 and B4 (cheeses B3A2 and B4A2). On the other hand, the most sensitive *L. acidophilus* cultures were A1 (cheese B1A1) and A2 (cheeses B2A2 and B4A2) since the decrease in viable cell counts during storage of 60 d was approximately 0.7, 0.8, and 0.9 log orders, respectively.

The viability of bifidobacteria and *L. casei* in BC-cheeses is presented in Table 2. In every case the probiotic bacteria counts were higher than 10^6 cfu/g during storage. Although a significant difference ($P < 0.05$) was found for *Bifidobacterium* sp. B5 in the cheese B5C2, it was lower than 1 log order. In BC-cheeses, *L. casei* cultures demonstrated survivability higher than bifidobacteria.

Table 3 shows the viable counts of bifidobacteria, *L. acidophilus*, and *L. casei* in BAC-cheeses. Although a significant difference ($P < 0.05$) was detected for the colony counts of *B. longum* B1 in the cheese B1A1C2 after 60 d of refrigerated storage, the population of viable cells was acceptable for a probiotic dairy product. In all other cases, no significant differences ($P > 0.05$) were detected and the viable cell levels were higher than 10^7 cfu/g during the 60 d the viability was monitored. The combination of cultures B1A1C2 was not suitable because their respective counts decreased during storage (1.3, 0.3, and 0.5 log orders, respectively, after 60 d). The combination of probiotic cultures B4A2C1 was the most interesting, since it allowed us to obtain the highest probiotic counts without visible negative interactions among the strains.

The initial contents of the acidifying bacteria in BA, BC, and BAC-cheeses ranged from 4.30 to 5.78 log orders (*Lactococcus lactis* A6) and from 8.46 to 9.33 log orders (*Streptococcus thermophilus* A4). In general, it was shown that starter bacteria counts lowered during storage. Significant differences ($P < 0.05$) in the colony counts were detected for *L. lactis* A6 in the cheeses B5A1, B5A2, B3A2, and B2A2C2, where the fall in cell counts ranged from 0.3 to 1.7 log orders. The low values for the cell counts of *L. lactis* A6 (<6 log orders) in all the cheeses might be because it was involved with a thermophilic bacteria (*S. thermophilus*) in a process that includes a stage of milk acidification at 44°C. However, its use was important because it improved cheese texture. For *S. thermophilus*, significant differences ($P < 0.05$) were observed in the cheeses B1A1, B1A2, and B4A2, but the fall in cell counts was always less than 1 log order. In all other cases, populations of viable cells higher than 10^9 cfu/g were obtained, except for cheeses B5A2 (30 d), B4A1 (60 d), and B5C2 (60 d) in which cases the counts ranged from 10^8 to 10^9 cfu/g.

Counts of coliforms, yeasts, and molds, carried out at 0, 30, and 60 d of refrigerated storage, were always lower than 10^3 cfu/g (data not shown).

Figure 1 shows the cell viability at pH 2 and 3 of *B. bifidum* B4, *L. acidophilus* A2, *L. casei* C1, *L. lactis* A6,

Table 1. Viability (log cfu/g) of bifidobacteria and *Lactobacillus acidophilus* in Fresco cheese type BA[1] (n = 6).

Strain combination	Count of bifidobacteria at (days)			Count of *L. acidophilus* at (days)		
	0	30	60	0	30	60
B1A1	7.07[a] ± 0.57	ND[2]	7.09[a] ± 0.53	7.50[c] ± 0.40	ND	6.76[c] ± 0.63
B1A2	6.91[a] ± 0.27	6.43[a] ± 0.21	6.56[a] ± 0.49	7.35[c] ± 0.15	7.15[c] ± 0.12	7.11[c] ± 0.03
B5A1	6.97[a] ± 0.16	6.91[a] ± 0.21	6.93[a] ± 0.13	ND	6.77[c] ± 0.15	6.80[c] ± 0.12
B5A2	7.55[a,b] ± 0.08	7.32[a] ± 0.08	7.59[b] ± 0.10	8.69[c] ± 0.15	8.51[c] ± 0.16	8.44[c] ± 0.28
B2A1	6.39[a] ± 0.55	6.43[a] ± 0.37	5.86[a] ± 0.46	6.03[c] ± 0.60	ND	5.56[c] ± 0.45
B2A2	6.48[a] ± 0.67	6.94[a] ± 0.14	6.80[a] ± 0.54	8.12[c] ± 0.16	8.46[c] ± 0.20	7.29[d] ± 0.34
B3A2	7.68[a] ± 0.17	7.07[b] ± 0.03	6.89[b] ± 0.16	7.40[c] ± 0.35	7.30[c] ± 0.22	7.16[c] ± 0.18
B4A1	7.72[a] ± 0.03	7.19[b] ± 0.11	7.39[b] ± 0.10	7.69[c] ± 0.30	7.34[c] ± 0.15	7.75[c] ± 0.14
B4A2	7.83[a] ± 0.13	7.90[a] ± 0.16	7.01[b] ± 0.15	7.94[c] ± 0.51	7.55[c] ± 0.46	7.01[c] ± 0.16

[a,b,c,d]Means in row with a common superscript do not differ significantly ($P > 0.05$).

[1]BA: *Bifidobacterium bifidum* and *L. acidophilus*.

ND: Not done.

and *S. thermophilus* A4 in BAC-cheese homogenates, during 3 h at 37°C, partially simulating stomach acidity. *S. thermophilus* A4 was clearly the most sensitive to both pH values; it was not detected after 30 min. *Lactobacillus lactis* A6 had almost the same behavior under both acidic conditions, falling approximately 3 log orders after 3 h. Among the probiotic bacteria, *L. casei* C1 was the most sensitive at pH 2, followed by *L. acidophilus* A2, even though their decrease in cell counts was almost identical after 3 h (near 5.2 log orders). At pH 3, both of them behaved approximately the same, and their population was reduced by only 1.3 log orders. *Bifidobacterium bifidum* B4 was the probiotic bacteria most resistant to the acidic environment, since the diminution of its viable cell counts was negligible at pH 3 and lower than 2 log orders at pH 2 after 3 h. In control experiments (data not shown), the survivability of the lactic and probiotic cultures was lower than that recorded in the cheese assays. At pH 2, cultures of *L. acidophilus* A2 and starter bacteria were not able to survive for 1 h, while counts of *B. bifidum* B4 and *L. casei* C1 lowered 5 and 7 log orders, respectively. At pH 3, the starter bacteria were not even able to survive after 1 h. *L. casei* C1 was the most resistant culture because its counts decreased 2 log orders after 3 h. The other probiotic cultures had lowered acidic resistance; their levels decreased 3.5 (*B. bifidum* B4) and 6.5 (*L. acidophilus* A2) log orders after 1 h.

DISCUSSION

The potential health-promoting affects of probiotic bacteria have stimulated research in recent years (20, 21, 28) to optimize the industrial production of dairy products containing them. To date, the most important popular vehicles for probiotic bacteria have been fermented dairy foods, such as yogurts and other fermented milks (16, 17, 24). To expand the probiotic product spectrum, some efforts have been made recently in Argentina to produce cheeses with probiotic cultures.

With this aim, we assayed the viability of nine different combinations of bifidobacteria (five cultures) and *L. acidophilus* (two cultures) in Argentinian Fresco cheese. All of them performed well; the decrease in counts of both organisms after 60 d of refrigerated storage was, in every case, less than 1 log order.

The use of bifidobacteria in combination with *L. casei* was not previously reported for probiotic cheese production. The survival of both bacteria in Argentinian Fresco cheese was also satisfactory. After 60 d of storage, the decreases in colony counts were lower than 1 log order for bifidobacteria and null for *L. casei*. These results demonstrated that *L. casei* cultures were more adaptable to the Fresco cheese environment. Stanton et al. (31) reported that *L. paracasei* was also satisfactorily viable during the long ripening period of Cheddar cheese.

Table 2. Viability (log cfu/g) of bifidobacteria and *Lactobacillus casei* in Fresco cheese type BC[1] (n = 6).

Strain combination	Count of bifidobacteria at (days)			Count of *L. casei* at (days)		
	0	30	60	0	30	60
B5C2	7.32[a] ± 0.16	7.21[a] ± 0.12	6.57[b] ± 0.19	7.04[c] ± 0.22	7.19[c] ± 0.31	7.00[c] ± 0.16
B4C1	8.60[a] ± 0.02	8.42[a] ± 0.53	8.15[a] ± 0.35	7.23[c] ± 0.11	7.16[c] ± 0.20	7.33[c] ± 0.53

[a,b,c,d]Means in row with a common superscript do not differ significantly ($P > 0.05$).

[2]BC: Bifidobacteria and *L. casei*.

Table 3. Viability (log cfu/g) of bifidobacteria, *Lactobacillus acidophilus* and *Lactobacillus casei* in Fresco cheese type BAC[1] (n = 6).

Strain combination	Count of bifidobacteria at (days)			Count of *L. acidophilus* (days)			Count of *L. casei* (days)		
	0	30	60	0	30	60	0	30	60
B1A1C2	$7.56^a \pm 0.11$	$7.14^a \pm 0.31$	$6.30^b \pm 0.12$	$8.32^c \pm 0.17$	$8.27^c \pm 0.19$	$8.04^c \pm 0.23$	$8.40^d \pm 0.29$	$8.34^d \pm 0.15$	$7.90^d \pm 0.24$
B2A2C2	$7.24^a \pm 0.13$	$7.16^a \pm 0.11$	$7.29^a \pm 0.08$	$7.75^c \pm 0.18$	$7.35^c \pm 0.30$	$7.70^c \pm 0.07$	$7.57^d \pm 0.22$	$7.34^d \pm 0.34$	$7.60^d \pm 0.36$
B4A2C1	$8.71^a \pm 0.13$	$8.62^a \pm 0.25$	$8.58^a \pm 0.26$	$8.39^c \pm 0.22$	$8.46^c \pm 0.33$	$8.56^c \pm 0.26$	$8.33^d \pm 0.21$	$8.61^d \pm 0.29$	$8.66^d \pm 0.25$

[a,b,c,d]Means in column with a common superscript do not differ significantly ($P > 0.05$).

[1]BAC: Bifidobacteria, *L. acidophilus* and *L. casei*.

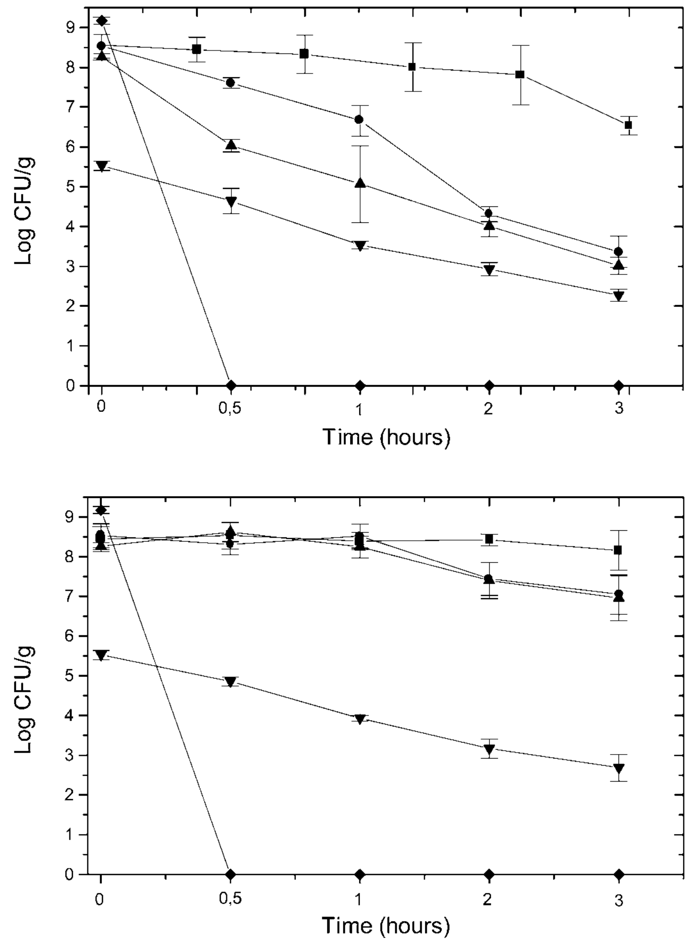

Figure 1. Viability of *Bifidobacterium bifidum* B4 (■), *Lactobacillus acidophilus* A2 (●), *Lactobacillus casei* C2 (▲), *Lactobacillus lactis* A6 (▼) and *Streptococcus thermophilus* A4 (◆) in a BAC-cheese homogenate, HCl-acidified (pH 2, top, and pH 3, bottom) at 37°C. The values are the mean of three determinations.

The simultaneous addition of bifidobacteria, *L. acidophilus*, and *L. casei* to cheeses has not been reported previously. When bifidobacteria (three cultures), *L. acidophilus* (two cultures), and *L. casei* (two cultures) were used in three different combinations as probiotic adjuncts in Fresco cheese, the results showed good viability during 60 d of storage. Although the probiotic counts were always higher than 10^6 cfu/g, the probiotic mixture B1A1C2 showed a relative low adaptability to Fresco cheese, especially for *B. longum* B1. The combination B4A2C1 was chosen for the industrial development of Argentinian Fresco cheese because it allowed us to obtain the highest counts for probiotic bacteria and excellent cell viability up to 60 d of storage.

On the other hand, the lactic starter microflora (*L. lactis*/*S. thermophilus*) had, as was expected, normal survival in the cheese. *L. lactis* and *S. thermophilus* counts were in every case higher than 4 and 8 log orders, respectively. Furthermore, the growth of contaminant microflora (coliforms and yeasts and molds) was stopped at levels lower than 10^3 cfu/g in every case.

Besides being able to survive satisfactorily in a food carrier, probiotic organisms must not adversely affect carrier composition, texture, or flavor, and they must also be capable of tolerating the conditions encountered during their transit through the gastrointestinal tract. Here, the low pH of the stomach is undoubtedly one of the main barriers to overcome (3, 18, 31). In this study, we evaluated "in vitro" the suitability of Argentinian Fresco cheese to go through that hydrochloric medium and deliver viable probiotic cells into the small intestine. When the probiotic cultures (B4A2C1) were added to the cheese, they demonstrated an excellent ability to remain viable up to 3 h in a cheese homogenate at pH 3. At pH 2, the cell viability of the cultures was more affected; *B. bifidum* B4 was the most resistant probiotic organism. The survivability of pure probiotic cultures in hydrochloric solutions was significantly lower than that when the bacteria were incorporated into the cheese. This demonstrated the protective effect of the cheese as probiotic carrier under cheese homogenate at pH 3 conditions.

CONCLUSIONS

This study showed that Argentinian Fresco Cheese can be used as an adequate carrier of probiotic bacteria. The cultures of bifidobacteria, *L. acidophilus*, and *L. casei* used in combination demonstrated satisfactory survival during 60 d. In all cases, final numbers of viable cells were still above the levels suggested to produce their claimed health benefits. In addition, when the probiotic cheese and pure probiotic cultures were suspended in hydrochloric solutions to partially simulate the stomach environment, the cell viability of probiotic organisms was not or slightly affected only in the first case.

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