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ORIGINAL ARTICLE

Cell viability and immunostimulating and protective capacities of *Bifidobacterium longum* 5^{1A} are differentially affected by technological variables in fermented milks

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Keywords
animal survival, bifidobacteria, fermented milks, immunostimulation, *Salmonella* infection, storage period, viability.

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Abstract

Aim: To investigate the cell viability of *Bifidobacterium longum* 5^{1A} in fermented milks and to study its immunostimulating and protective capacity against *Salmonella enterica* ssp. *enterica* serovar Typhimurium infection in mice.
Methods and Results: *Bifidobacterium longum* 5^{1A} was added to milk fermented with different yoghurt starter cultures, before or after fermentation, and viability was monitored during storage (5°C, 28 days). Resistance to simulated gastric acid digestion was assessed. Fermented milks were orally administered to mice for 10 days followed by oral infection with *Salmonella* Typhimurium. The number of IgA+ cells in the small and large intestine was determined before infection. Survival to infection was monitored for 20 days. *Bifidobacterium longum* 5^{1A} lost viability during storage, but the product containing it was effective for the induction of IgA+ cells proliferation in the gut and for the protection of mice against *Salm.* Typhimurium infection.
Conclusions: Cell viability of *Bif. longum* 5^{1A} in fermented milks along storage did not condition the capacity of the strain to enhance the number of IgA+ cells in the gut and to protect mice against *Salmonella* infection.
Significance and Impact of the Study: The uncoupling of cell viability and functionality demonstrated that, in certain cases, nonviable cells can also exert positive effects.

Introduction

Several definitions of probiotic bacteria have been proposed already, but the one with the prevailing international scientific consensus is the definition adopted in 2002 by the Joint Committee of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), which establishes that probiotics are 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2002). As pointed out by this definition, the capacity of probiotics to exert a beneficial effect on the consumer's health is obtained when they are incorporated into a food in a given concentration as viable cells. For this reason, the microbiological control of cell viability and quantity is routinely used in the food industry for the monitoring of their presence in food, as functional criterion. Depending on the countries, the scientific criteria (Champagne and Gardner 2005; Ross *et al.* 2005) or the local legislations (Amagase 2008; Degnan 2008; Saxelin 2008) require different concentrations of viable bacteria to guarantee the functional quality of the food and to call it, then, a probiotic food. However, on the one hand, health benefits are also, at least to a certain extent, recognized to nonviable cells (Ouwehand and Salminen 1998; Kataria *et al.* 2009). On the other hand, in the last years, there has been evidence of certain changes in functionality, that is, in the qualitative and quantitative magnitude of the

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beneficial effect of a strain, without levels of viable cells being modified (Saarela *et al.* 2006; Grzeskowiak *et al.* 2011; Vinderola *et al.* 2011a). In this context, the microbiological count of viable cells would only partially reflect the functional capacity of the strain. Therefore, it is necessary to know, to manage and to control the microbiological and technological variables involved in food production that may alter the functionality of a strain in a particular food matrix. The aim of this work was to study the cell viability and functionality of *Bifidobacterium longum* 5^{1A} in fermented milks manufactured under different technological conditions.

Materials and methods

Strains, starter cultures and culture conditions

Bifidobacterium longum 5^{1A} was used. The strain had been isolated from a healthy child, identified by molecular tools and kept at the Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (Belo Horizonte, Brazil). This strain demonstrated to be effective against constipation in children in a human clinical trial (Guerra *et al.* 2011). The strain was maintained in de Man, Rogosa and Sharp (MRS; Biokar, Beauvais, France) broth + 20% (v/v) glycerol at −70°C. Before use, the strain was cultured at least three times in MRS broth supplemented with 0·1% (w/v) L-cysteine hydrochloride (Biopack, Buenos Aires, Argentina) during 18 h under anaerobic conditions (Oxoid, Basingstoke, UK) at 37°C. For the preparation of a concentrated frozen stock, an overnight culture was centrifuged (6000 ***g***, 15 min, 5°C), washed twice with phosphate-buffered saline (PBS) solution (pH 7·4) and resuspended in 20% (w/v) skim milk (San Regim, Buenos Aires, Argentina). Cell suspensions were distributed in 2 ml aliquots and immediately frozen at −70°C until use. Cell counts on agar plates (MRS + 0·1% (w/v) of L-cysteine hydrochloride, 37°C, anaerobic incubation, 48 h) were performed before and after freezing to verify whether any lost in cell viability was produced. For fermented milk production, the following direct vat system (DVS) yoghurt starter cultures (containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, in an undetermined cocci:rod rate) were used according to the manufacturer's instruction (Chr. Hansen): Yo-Flex 702 (frozen culture), Yo-Flex 812, Yo-Flex 903 and Harmony (freeze-dried cultures). For the pathogenic challenge in mice, a *Salmonella enterica* ssp. *enterica* serovar Typhimurium strain of human origin isolated at the Fundação Ezequiel Dias (FUNED, Belo Horizonte, Brazil) was used. *Salmonella* was grown overnight (aerobic incubation, 37°C) in Brain Heart Infusion (BHI) broth (Britania, Buenos Aires, Argentina).

Fermented milk productions

Two different fermented milk productions were performed in duplicate in independent assays on different days. In the first assay, 10% (w/v) bovine skim milk was inoculated with the starter culture Yo-Flex 702 and a fresh or frozen culture of *Bif. longum* 5^{1A} to attain an initial cell count of about 7·5–8·0 log colony forming units (CFU) ml^{-1}. The addition of fresh or frozen bifidobacteria was done before or after milk fermentation. For another samples, 5% inulin (Benero-Orafti, Aandorenstraat, Belgium) was added before milk autoclaving (110°C for 30 min). Milk was fermented at 43°C until pH reached a value of 4·60. When bifidobacteria were added after milk fermentation, the fermented product was refrigerated to 5°C in a water bath before bifidobacteria addition. The fermented milks manufactured in the first assay are shown in Table 1.

For the second assay of fermented milk production, 12% (w/v) bovine skim milk added of 2% (w/v) whey protein concentrate (Arla Foods Ingredients S.A., Buenos Aires, Argentina) was used. Milk was inoculated with the starter cultures Yo-Flex 812, Yo-Flex 903 or Harmony and a frozen culture of *Bif. longum* 5^{1A} to attain an initial cell count of bifidobacteria of about 7·5–8·0 log CFU ml^{-1}. The addition of bifidobacteria was done before or after

Table 1 Cell viability (log CFU ml^{-1} ± SD) of *Bifidobacterium longum* 5^{1A} in different types of fermented milks, and their corresponding pH, during the refrigerated storage at 5°C

Type of fermented milk (type of culture/moment of inoculation of the bifidobacteria)	Log CFU ml^{-1} ± SD pH ± SD Storage day		
	0*	14	28
Fresh culture/before fermentation	6·46 ± 0·12^{a} 4·63 ± 0·08^{a}	5·44 ± 0·16^{b} 4·68 ± 0·03^{a}	<3·00 4·73 ± 0·02^{a}
Fresh culture/after fermentation	8·03 ± 0·14^{a} 4·59 ± 0·01^{a}	6·60 ± 0·28^{a} 4·65 ± 0·04^{a}	<3·00 4·64 ± 0·10^{a}
Frozen culture/before fermentation	6·56 ± 0·37^{a} 4·59 ± 0·09^{a}	5·82 ± 0·13^{b} 4·68 ± 0·05^{a}	<3·00 4·68 ± 0·09^{a}
Frozen culture/after fermentation	8·19 ± 0·11^{a} 4·60 ± 0·01^{a}	6·43 ± 0·28^{b} 4·70 ± 0·06^{a}	<3·00 4·66 ± 0·12^{a}
Fresh culture/after fermentation/inulin	8·17 ± 0·12^{a} 4·55 ± 0·03^{a}	6·61 ± 0·23^{b} 4·52 ± 0·12^{a}	<3·00 4·56 ± 0·07^{a}
Frozen culture/after fermentation/inulin	8·22 ± 0·08^{a} 4·59 ± 0·04^{a}	6·13 ± 0·09^{b} 4·58 ± 0·05^{a}	<3·00 4·59 ± 0·11^{a}

Values in rows with different superscript are significantly different ($P < 0·05$).

*Cell counts of bifidobacteria at day 0 were performed immediately after fermentation (in case bifidobacteria where added before milk fermentation) or immediately after bifidobacteria addition (in case bifidobacteria were added after milk fermentation).

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milk fermentation, and milk was fermented at 40°C, until pH reached a value of *c.* 4·60. Bifidobacteria addition after milk fermentation was carried out as stated before. The fermented milks manufactured in the second assay are shown in Table 3. Cell viability of bifidobacteria in both assays was monitored periodically during the refrigerated storage at 5°C for 28 days by selective agar plate counts (MRS-LP: MRS + 0·2% (w/v) lithium chloride + 0·3% (w/v) sodium propionate, 37°C, anaerobic incubation, 48 h) as described by Vinderola and Reinheimer (1999).

Resistance of *Bifidobacterium longum* 5^{1A} in fermented milks to simulated gastric digestion

A volume (5 ml) of fermented milk sample (from the first assay) was mixed with the same volume of a simulated 'saliva-gastric' solution containing $CaCl_2$ (0·22 g l^{-1}), NaCl (16·2 g l^{-1}), KCl (2·2 g l^{-1}), $NaHCO_3$ (1·2 g l^{-1}) and 0·3% (w/v) porcine pepsin (Merck, Darmstadt, Germany) and adjusted to pH 3·00 with 5 N and 1 N HCl (Vinderola *et al.* 2011b). One-millilitre samples were taken immediately after mixture (before pH adjustment) and after 90 min of incubation at 37°C in a water bath for bifidobacteria cell count (MRS-LP, 37°C, anaerobic incubation, 48 h). Results were expressed as the difference in cell counts (log CFU ml^{-1}) at time zero and after 90 min of incubation. Resistance to simulated gastric digestion was evaluated every 2 weeks during the storage at 5°C.

In vivo assays

Animals.

A total of 70 male BALB/c mice (19–21 g) were obtained from the random bred (in-bred) colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). Animals were allowed to stand at the INLAIN animal facility for a week before starting the assays. Animals were housed together in plastic cages (four animals per cage) and kept in a controlled environment at 21 ± 2°C with humidity at 55 ± 2%, with a 12-h light/dark cycle. Mice were maintained and treated according to the guidelines of the National Institute of Health (NIH, USA). The experiments with animals were approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina).

Experimental design for feeding procedures.

Mice were divided into five groups (14 animals per group: four animals for IgA determination and ten animals for the infection assay) that received for ten consecutive days the following products by oral gavage (0·2 ml per mouse per day): (i) 10% (w/v) bovine skim milk; (ii) milk fermented by the starter culture Yo-Flex 702 (without addition of bifidobacteria added); (iii) fermented milk (starter culture Yo-Flex 702) containing bifidobacteria added as a fresh culture after milk fermentation; (iv) fermented milk containing bifidobacteria added as a frozen culture after milk fermentation and (v) fermented milk + inulin + bifidobacteria added as a fresh culture after milk fermentation. All animals received, simultaneously and *ad libitum*, tap water and a sterile conventional balanced diet (Cooperación, Buenos Aires, Argentina) containing proteins, 230 g kg^{-1}; raw fibre, 60 g kg^{-1}; total minerals, 100 g kg^{-1}; Ca, 13 g kg^{-1}; P, 8 g kg^{-1}; water, 120 g kg^{-1} and vitamins.

Evaluation of functionality I: IgA-producing in the intestinal lamina propria.

After the 10-day feeding period, four animals of each group were injected intraperitoneally with an anaesthetic mixture containing nine parts of ketamine (100 mg ml^{-1}), nine parts of xylazine (20 mg ml^{-1}), three parts of acepromazine (10 mg ml^{-1}) and 79 parts of sterile saline solution. Animals were sacrificed by cervical dislocation. Liver was removed and homogenized in 5 ml of sterile PBS. One millilitre of liver homogenate was pour-plated onto MacConkey agar (safety assay: translocation of enterobacteria to liver). Plates were incubated at 37°C for 24 h in aerobiosis. The small and large intestines were removed for histological studies according to Vinderola *et al.* (2005). Paraffin-sections (4 mm) were stained with haematoxylin-eosin and then evaluated by light microscopy examination (double-blind observations). The number of IgA-producing (IgA+) cells was determined on histological slices of samples from the ileum near Peyer's patches and from the large intestine. The immunofluorescence test was performed using α-chain-specific anti-mouse IgA fluorescein isothiocyanate (FITC) conjugate (Sigma, St Louis, MO). Histological slices were deparaffinized and rehydrated in a series of decreasing ethanol concentrations (from absolute ethanol until 70° alcohol). Deparaffinized histological samples were treated with a dilution (1/100) of the antibody in PBS and incubated in the dark for 30 min at 37°C. Then, samples were washed twice with PBS and examined using a fluorescent light microscope (Nikon Eclipse using a Hg lamp). The results were expressed as the number of positive cells (fluorescent cells)/10 fields. Positive (fluorescent) cells were counted with a magnification of 400× (double-blind counts). Data were reported as the mean of three counts (each one in a different histological slice) for each animal.

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Evaluation of functionality II: protective capacity against Salmonella enterica serovar Typhimurium infection.
After the 10-day feeding period with fermented milks, the remaining ten animals of each group were orally challenged with a single infective dose (10^6 CFU per mouse) of *Salm. enterica* serovar Typhimurium cultured overnight as stated before and washed twice with PBS. Animal survival was monitored during 20 days postinfection. In this period, mice also received the fermented products. The same batch of fermented milk was used along feeding. Results were expressed as the percentage of animals that survived the infection.

Statistical analysis

Technological experiments were carried out in two independent assays in duplicate. Data were analysed using the one-way ANOVA procedure of SPSS software (SPSS Inc., Chicago, IL, USA). The differences between means were detected by the Duncan's Multiple Range Test. Cumulative mortality and mortality evolution results were submitted to Fisher Exact Test and Survival Log Range Test, respectively. Data were considered significantly different when $P < 0{\cdot}05$.

Results

Cell viability of bifidobacteria in fermented milks

Two productions of fermented milks were carried out, exploring different technological variables commonly used in the fermentative industry to find a suitable food matrix for *Bif. longum* 5^{1A}. In the first assay, fermented milk was produced using 10% (w/v) skim milk and a temperature of fermentation of 43°C. Yo-Flex 702 was used as starter culture, and bifidobacteria were added before or after milk fermentation, as a fresh or frozen culture. The effect of inulin on the protection of cell viability during storage was also assessed. In every case, pH reached the desired value in a range of time of 4·5–5·5 h. Table 1 shows the cell counts of bifidobacteria and pH during 28 days of refrigerated storage. It can be noticed that no postacidification during storage occurred and pH values were maintained at around 4·60. With respect to cell viability, a maximum of 2-log order of cell viability decay was observed by day 14 of storage, being cell counts lower to 3 log cycles by the end of the storage period in every case. When bifidobacteria were added to milk before fermentation, a *c.* 1·5-log order of cell viability decay was already observed by the end of the fermentation. However, in any case, cell viability diminished through the storage period, reaching values lower than 3 log units by the end of the refrigerated storage period, even in the presence of inulin.

The resistance of bifidobacteria to simulated gastric digestion was assessed at three point intervals during storage. After fermentation, sensitivity to simulated gastric digestion was higher for cells inoculated before fermentation (cell viability decays from 1·30 to 1·70 log cycles) compared to cultures added to milk already fermented (cell decays ranging from 0·43 to 0·99 log cycles) (Table 2). Cell sensitivity to simulated gastric acidity increased during storage with loss in cell viability ranging from 3·00 to 5·25 log cycles CFU ml^{-1} after 14 days of storage, reaching values higher than 3 log cycles by day 28 of storage.

The second assay was designed to try to increase cell viability by modifying some technological variables known as potential inhibitors of bifidobacteria viability in fermented milks. The three following strategies were tested: (i) total milk solids were increased from 10% (w/v) to 14% (w/v) by the addition of extra skim milk (2% w/v) and whey protein concentrate (2% w/v); (ii) fermentation temperature was lowered to 40°C; (iii) three different yoghurt starters were used. Under these conditions, a lower decrease in cell viability was observed during fermentation (time 0 of storage), but anyway cell counts gradually diminished in all cases during storage (Table 3). Indeed, higher cell counts of bifidobacteria were observed in the first assay by day 14 of storage (Table 1) compared to the second assay (Table 3). A slight acidification during storage occurred when starter cultures Yo-Flex 812 and 903 were used.

Cell functionality of bifidobacteria in fermented milks

We aimed at studying the *in vivo* effects of the oral administration to mice of the fermented milks manufactured in the first assay. The proliferation of IgA-producing cells and the protective capacity against *Salmonella*

Table 2 Resistance of *Bifidobacterium longum* 5^{1A} to simulated gastric acid digestion in different types of fermented milks during the refrigerated storage at 5°C

Type of fermented milk (type of culture/moment of inoculation of the bifidobacteria)	Δ log CFU ml^{-1}* Storage day 0	14	28
Fresh culture/before fermentation	1·30	3·58	>3·00
Fresh culture/after fermentation	0·65	5·25	>3·00
Frozen culture/before fermentation	1·70	3·00	>3·00
Frozen culture/after fermentation	0·51	5·25	>3·00
Fresh culture/after fermentation/inulin	0·43	5·18	>3·00
Frozen culture/after fermentation/inulin	0·99	4·70	>3·00

*Differences in cell counts (log CFU ml^{-1}) before and after acidification of the fermented milk to pH 3·0 for 90 min at 37°C in the presence of 0·5% NaCl and 0·1% porcine pepsin.

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Table 3 Cell viability (log CFU ml^{-1} ± SD) of *Bifidobacterium longum* 5^{1A} in fermented milks, and their corresponding pH, manufactured with different starter cultures (812, 903, Harmony) during the refrigerated storage at 5°C

Type of fermented milk (starter culture/inoculation of bifidobacteria)	Log CFU ml^{-1} ± SD pH ± SD Storage day				
	0*	7	14	21	28
812/before fermentation	7·07 ± 0·21^a	5·42 ± 0·01^b	3·08 ± 0·11^c	3·24 ± 0·65^c	<3·00
	4·64 ± 0·01^a	4·51 ± 0·03^b	4·56 ± 0·09^b	4·52 ± 0·02^b	4·54 ± 0·01^b
812/after fermentation	7·40 ± 0·08^a	5·78 ± 0·18^b	3·40 ± 0·57^c	<3·00	<3·00
	4·74 ± 0·01^a	4·66 ± 0·02^b	4·69 ± 0·06^b	4·68 ± 0·07^b	4·67 ± 0·01^b
903/before fermentation	7·34 ± 0·15^a	4·59 ± 0·83^b	<3·00	<3·00	<3·00
	4·66 ± 0·04^a	4·46 ± 0·01^b	4·45 ± 0·08^b	4·43 ± 0·06^b	4·42 ± 0·01^b
903/after fermentation	6·72 ± 0·26^a	4·90 ± 0·84^b	3·19 ± 0·27^c	3·00 ± 0·42^c	<3·00
	4·72 ± 0·03^a	4·49 ± 0·03^b	4·47 ± 0·08^b	4·44 ± 0·07^b	4·43 ± 0·01^b
Harmony/before fermentation	7·50 ± 0·36^a	5·02 ± 2·85^b	<4·00	<3·00	<3·00
	4·65 ± 0·01^a	4·71 ± 0·02^a	4·69 ± 0·06^a	4·69 ± 0·04^a	4·69 ± 0·02^a
Harmony/after fermentation	7·19 ± 0·24^a	5·00 ± 2·84^b	<4·00	<3·00	<3·00
	4·69 ± 0·08^a	4·84 ± 0·05^a	4·68 ± 0·16^a	4·67 ± 0·16^a	4·67 ± 0·25^a

Values in rows with different superscript are significantly different ($P < 0·05$).
*Cell counts of bifidobacteria at day 0 were performed immediately after fermentation (in case bifidobacteria where added before milk fermentation) or immediately after bifidobacteria addition (in case bifidobacteria were added after milk fermentation).

infection were chosen as indicators of functionality. The oral feeding of animals (for ten consecutive days) began the day after fermented milks were produced (in the first assay), and the same batch of fermented milks was used. The oral administration of the fermented milks induced no undesirable side effects as demonstrated by the absence of both translocation of enterobacteria to the liver and undesired morphological changes in the architecture of the small and large intestines by comparison with control mice (data not shown).

Figure 1 shows the number of IgA+ cells in the small and large intestine lamina propria of mice that received the fermented products. A significant increase ($P < 0·05$) in this parameter was observed in the small intestine of mice that received fermented milk containing bifidobacteria added, after fermentation, as a fresh or frozen culture or in animals that received fermented milk containing inulin and bifidobacteria. No significant differences were observed among these three groups of mice. In the large intestine, a significant increase in the number of IgA+ cells was observed when animals received fermented milks, with or without inulin, with bifidobacteria added as a fresh culture. No significant differences were observed between these two groups.

The other parameter of functionality assessed in this study was the capacity of the fermented products to protect the animals against an intestinal infection by *Salm.* Typhimurium. The pathogen was administered to mice as a single infective dose after the 10-day feeding

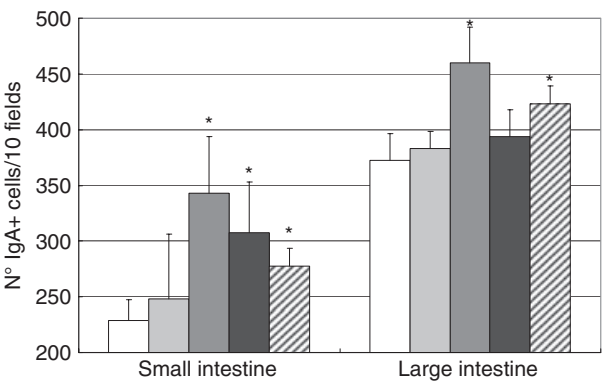

Figure 1 Effect of the oral administration of fermented milk containing *Bifidobacterium longum* 5^{1A} on the number of IgA-producing cells in the small and large intestine lamina propria of mice. Mice received 10% skim milk (control group, white columns), fermented milk without bifidobacteria (light grey column), fermented milk with bifidobacteria added as a fresh (grey column) or frozen (dark grey column) culture after fermentation or fermented milk + inulin, added with bifidobacteria as a fresh culture after fermentation (stripped column). * Significantly different compared with the control group ($P < 0·05$). ($N = 4$).

period with fermented milks. The survival postinfection was monitored for 20 days. Only animals that received (before and after infection) the fermented milk containing bifidobacteria added as a fresh culture without inulin showed a significant ($P = 0·012$) enhanced survival (100%) compared to the control group (50%) (Fig. 2).

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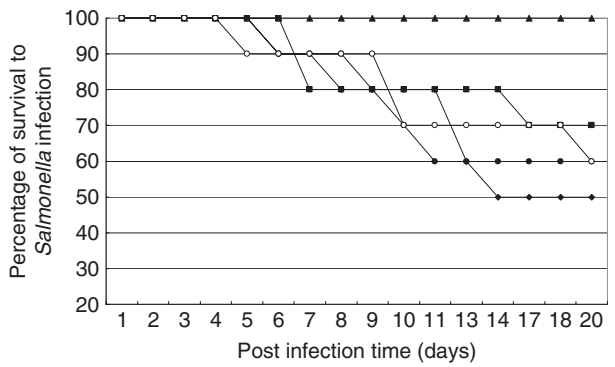

Figure 2 Effect of the oral administration of fermented milk containing *Bifidobacterium longum* 5^{1A} on the survival capacity of mice to an infection by *Salmonella enterica* serovar Typhimurium. Mice received 10% skim milk (control group, ◆), fermented milk without bifidobacteria (■), fermented milk with bifidobacteria added as a fresh (▲) or frozen (●) culture after fermentation or fermented milk + inulin, added with bifidobacteria as a fresh culture after fermentation (○). (N = 10).

Discussion

Bifidobacteria are normal inhabitants of the human gut, and only positive health's benefits have been ascribed to them in relation to their metabolic activity in the gut as indigenous micro-organisms (Leahy *et al.* 2005). Many bifidobacteria strains have been also isolated and characterized, from functional and technological points of view to use them as probiotics for humans (Picard *et al.* 2005), being fermented milks the main vehicles used for delivery in food (Vinderola *et al.* 2009). However, certain strains showed limited survival in fermented milks during refrigerated storage (Vinderola *et al.* 2009). Among bifidobacteria, some species seem to be more suitable for industrial applications than others. The good tolerance to both acidic and oxidative stress makes *Bifidobacterium animalis* ssp. *lactis* the most common species included in fermented dairy products, being able to maintain high levels of viable cells during product storage (Sánchez *et al.* 2010). For instance, *Bif. animalis* ssp. *lactis* BB-12 (Chr. Hansen) and DN-173 010 (Danone) are the most widely used strains in the food industry. However, other *Bifidobacterium* species were also successfully used for the development of commercial probiotic cultures and the following strains can be also found in the market: *Bifidobacterium bifidum* BF2, *Bifidobacterium breve* BR2, *Bifidobacterium infantis* BT and M-63, *Bif. longum* BG3 and BB536 and *Bifidobacterium pseudolongum* M-602 (Muller *et al.* 2009). In this study, a human-derived *Bif. longum* strain was used to study its performance in fermented milks. *Bifidobacterium longum* 5^{1A} is a strain isolated from the faeces of a healthy child that proved to be effective in the treatment of constipation in a recent human clinical trial (Guerra *et al.* 2011).

In a first assay, the capacity of *Bif. longum* 5^{1A} to withstand milk fermentation and the refrigerated storage of the product obtained was studied in the presence or absence of inulin, when the strain was added as a fresh or frozen culture before or after milk fermentation. The addition of bifidobacteria before or after fermentation was done to partially mimic the production of set or stirred fermented milks, respectively. It is worthy to mention that many studies of survival of probiotics in food are carried out using viable cells derived from fresh overnight cultures. However, in the fermentative food industry, probiotics are mainly commercialized as frozen or freeze-dried cultures (Muller *et al.* 2009). In all cases, *Bif. longum* 5^{1A} lost viability during milk fermentation and/or during the cold storage of the fermented milk obtained. Inulin was reported to enhance bifidobacteria survival during storage of fermented milks (Capela *et al.* 2006; Akın *et al.* 2007). However, in our study no positive effect was observed for inulin on cell viability during storage, showing the specific influence of strain and food matrix on the results. Additionally, cell sensitivity of bifidobacteria to simulated gastric acidity also increased during the cold storage of the fermented milks. In line with previous results, changes in resistance of probiotic bacteria to simulated gastric digestion during shelf life were reported for bifidobacteria in fruit juice and low-fat milk (Saarela *et al.* 2006) and for lactobacilli in commercial fermented milks (Vinderola *et al.* 2011b).

In the second assay, different yoghurt starter cultures were used, and milk contained a higher percentage of total solids and a lower fermentative temperature was applied. With these changes, a better survival of bifidobacteria was observed during fermentation. These factors (starter culture identity, total solids and fermentation temperature) taken together have been pointed out as candidate technological variables to manage and optimize survival of probiotic bacteria in fermented milks (Champagne and Gardner 2005; Ross *et al.* 2005). However, a better survival of bifidobacteria was not observed during the refrigerated storage of the fermented products manufactured. In this context, other technological variables should be further explored to find a suitable way for delivering this strain. For instance, *Bif. longum* 5^{1A} demonstrated to remain viable at levels of 9·0 log CFU mg^{-1} for at least 7 days in goat yoghurt (Guerra *et al.* 2011). Additionally, Mazochi *et al.* (2010) showed a survival of 7·5 log units (CFU ml^{-1}) of this strain for 40 days in yoghurt manufactured with goat milk and flavoured with strawberries. This fact shows that the food matrix in which probiotics are included has a decisive role in determining their viability and functionality during the

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manufacture of the product and protection of cells upon consumption (Ranadheera *et al.* 2010). For instance, cheeses may have some advantages over fermented milks as a delivery system for probiotics with sensitivity to lactic acidity, because of their higher pH, more solid matrix (oxygen exclusion) and higher fat content. Generally, pH of cheeses ranges from 4·7 to 5·6 whereas that of fermented milks may reach values as low as 3·5–3·7 (Vinderola *et al.* 2011c). Another tool to protect sensitive cells in the food matrix for better survival is the application of microencapsulation techniques, some of them already able to be scaled up for industrial applications (Burgain *et al.* 2011).

One of the health benefits ascribed to probiotic bacteria or food containing them is their capacity to positively modulate the gut immune response, in a strain-specific way (López *et al.* 2010). The activation of the intestinal immune system may lead to the exertion of different health effects in a therapeutic or preventive way, such as prevention of certain intestinal infections, inflammatory diseases or colon cancer (Yan and Polk 2010) or the resolution of different diarrheas (Sartor 2004). Within the gut mucosa, the main function of IgA is to exert immune exclusion of pathogens and foreign proteins in cooperation with the innate nonspecific defence mechanisms (MacPherson *et al.* 2001). In this study, fermented milks were orally administered to mice for ten consecutive days. Animals were then orally challenged with a single infective dose of *Salm.* Typhimurium, and survival to infection was monitored. The technological variables 'fresh' and 'frozen' bifidobacteria and the addition or not of inulin had a similar impact on the proliferation of IgA+ cells in the small intestine. However, in the large intestine, the addition of bifidobacteria as frozen cells fails to induce the proliferation of IgA+ cells compared to fresh cultures. The reason of this observation remains unknown for the moment, and further studies of the immune mechanisms involved should be conducted. However, and in line with these findings, we already observed that some technological treatments, such as spray-drying, may change the immunostimulating capacity of bioactive compounds (Burns *et al.* 2010). As many of the antigenic determinants presenting immunostimulating capacity are located on the surface of the cell wall in probiotic bacteria (Corthésy *et al.* 2007), they might have been somehow affected by the technological treatment applied (freezing in this case).

In relation to the survival capacity of mice to a pathogenic challenge after a previous treatment with the different fermented milks, only the group that received fermented milk containing bifidobacteria added as a fresh culture presented a significant higher survival capacity compared to the control group. To the light of these results, one might have expected that the group of mice that received fermented milk containing bifidobacteria as fresh culture and inulin could have had a similar survival capacity. However, this was not observed in the present study, and this result can be added to the various contradictory results on prevention of *Salmonella* infections by prebiotics, which have been published until now. As examples of negative data, Ten Bruggencate *et al.* (2003) reported that dietary fructo-oligosaccharides increase translocation of *Salmonella* in rats in a dose-dependent way, while Petersen *et al.* (2009) reported that supplementing a cornstarch-based rodent diet with 10% FOS or XOS increased the translocation of *Salm.* Typhimurium SL1344 in mice. On the other hand, there are many reports concerning the positive effects of inulin administration, alone or with probiotics, on prevention or attenuation of *Salmonella* infection in mouse models (Buddington *et al.* 2002; Apanavicius *et al.* 2007; Benyacoub *et al.* 2008; Rishi *et al.* 2009). Further studies are necessary to determine the role of inulin during the course of *Salmonella* infection in mouse models.

Conclusions

Even when *Bif. longum* 5^{1A} demonstrated to be sensitive to the fermented milks manufactured in terms of cell viability, the product containing this strain as a fresh culture proved to be effective for the induction of IgA+ cells proliferation in the small and large intestine and for the protection of mice against *Salm.* Typhimurium infection. The uncoupling of cell viability and functionality demonstrated that, in certain cases, nonviable cells can also exert positive effects. However, for industrial and quality control purposes, other technological variables must still be explored (goat milk, microencapsulation, addition to cheeses) to attain a high level of viable cells during shelf life, able to be verified by quality control procedures.

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