International Journal of Dairy Technology SDT SOCIETY OF DAIRY TECHNOLOGY

doi: 10.1111/1471-0307.12141

ORIGINAL RESEARCH

Influence of dairy practices on the capacity of probiotic bacteria to overcome simulated gastric digestion

PATRICIA BURNS,[†] LISANDRO LAFFERRIERE,[†] GABRIEL VINDEROLA* and JORGE REINHEIMER

Instituto de Lactología Industrial (INLAIN, UNL-CONICET), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santiago del Estero 2829, Santa Fe 3000, Argentina

The aim of this study was to evaluate the influence of microbiological and technological factors on the viability and functionality of probiotic Lactobacillus *strains. In particular, the influence of harvesting time, food matrix, refrigerated storage, time of inoculation and refrigerated storage in fermented milk on the resistance to simulated gastric digestion (RSGD) was evaluated. Moreover, strain resistance to simulated gastric digestion was compared to human gastric fluid. Results showed that the variables studied affected, in a different way and in a strain-dependent manner, the RSGD. No direct relation was observed between cell viability and RSGD.*

Keywords *Lactobacillus*, Probiotic, Storage, Resistance to gastric digestion, Functionality.

INTRODUCTION

Probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2002). Many micro-organisms are currently used as human probiotics, the strains most widely used in fermented dairy products belonging to the genus *Bifidobacterium* or to *Lactobacillus casei* and *Lactobacillus acidophilus* groups (Vinderola *et al.* 2011a). With regard to their viability and functionality of probiotic bacteria in fermented dairy products, this can be modified by many microbiological and technological factors, which should therefore be taken into account. In fact, the factors involved during biomass production of a strain (medium pH, available sugars and their concentrations, growth phase at harvesting, etc.), technological processing and the food matrix into which micro-organisms are added may significantly affect both their resistance to biological barriers (gastric acidity and bile salts) and their capacity to interact with immune cells, thus conditioning its functionality (Vinderola *et al.* 2011b). Viability can be considered as the amount of viable cells displayed by a culture under a given condition, whereas functionality is a more sophisticated concept comprising a group of nonexhaustive features, including viability, which confer on a probiotic strain the properties responsible for health improvement. However, as viability does not always mean full functionality, it is important to establish the difference between both parameters. Under certain conditions, bacteria can survive, although some of their functional characteristics such as resistance to low pH, adhesion to the intestinal epithelium or immunomodulating capacity may not be fully displayed, which could at least partially impair its capacity to exert a beneficial effect on health (Reilly and Gilliland 1999; Vinderola *et al.* 2011a). Saarela *et al.* (2006), for example, studied the stability of freeze-dried bifidobacteria in fruit juice and low-fat milk and found no changes in cell viability but lower acid and bile tolerance of cells during storage. Freeze-dried cells of *B. animalis* subsp. *lactis* INL1 showed higher tolerance to low pH (pH 2.0) when grown at pH 5.0 than when grown at pH 6.5 (Vinderola *et al.* 2012). Similar results had been reported by Saarela *et al.* (2009) for *L. rhamnosus* VTT E-97800. The appropriate choice of a probiotic micro-organism involves, as an essential feature, its ability to reach, survive and persist in the environment in which it is intended to act. As the large intestine is the site of action for most probiotic bacteria, for these bacteria to be effective they must arrive viable in sufficient

*Author for correspondence. E-mail: gvinde@fiq.unl.edu.ar.
[†]These authors contributed equally to this work.

© 2014 Society of Dairy Technology

numbers at the site of action. During passage through the gut, probiotic strains must overcome harsh environmental conditions. The acidic stomach environment and the presence of bile in the duodenum are major factors affecting the viability of probiotic bacteria (Mainville *et al.* 2005). Resistance to human gastric transit has been demonstrated *in vivo* for probiotic lactic acid bacteria and bifidobacteria, thus constituting an important *in vitro* selection criterion for probiotic micro-organisms (Zanoni *et al.* 2008). Consequently, a preliminary *in vitro* assessment becomes mandatory (FAO/WHO 2002). This assessment has traditionally paid special attention to the ecological origin of bacteria, their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere to intestinal surfaces (Morelli 2007). However, no international consensus exists so far about a standardised protocol to assess resistance to the conditions found in the gastrointestinal tract. The aim of this study was to evaluate the influence of technological factors on the functionality of *Lactobacillus* strains, measured as the resistance to simulated gastric digestion, as well as to review some of the various *in vitro* conditions reported in literature to perform a simple assessment of simulated gastric acid resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Four commercial strains, *L. casei* NAD, *L. acidophilus* DRU, *L. paracasei* A13 and *L. casei* BIO, were used in this study. Commercial names of the strains are not disclosed for confidentiality reasons. Strains were grown in MRS broth (Biokar Diagnostics, Beauvais, France) at 37 °C in aerobiosis. Bacterial stocks were kept at −70 °C in MRS + glycerol (20% v/v). In all experiments, strains were transferred three times (18 h, 37 °C, aerobiosis) to MRS broth before use. For fermented milk production, frozen stock cultures of *L. casei* NAD were prepared. Overnight cultures (18 h, 37 °C, aerobiosis) were washed twice with phosphate-buffered saline (PBS) solution (pH 7.2), resuspended in 20% (w/v) reconstituted skim milk (RSM) and frozen-stored at −70 °C.

Determination of resistance to simulated gastric digestion

The resistance to simulated gastric digestion (RSGD) was studied by an *in vitro* assay of gradual drop of pH suggested by Mainville *et al.* (2005), slightly modified. Acidification curves are shown in Figure 1. Samples (cell suspensions from overnight cultures or fermented milks) were mixed with the same volume of a 'saliva-gastric'-resembling solution containing $CaCl_2$ (0.22 g/L), NaCl (16.2 g/L), KCl (2.2 g/L), $NaHCO_3$ (1.2 g/L) (Marteau *et al.* 1997) and 0.6% (w/v) porcine pepsin (Merck, Darmstadt, Germany). Cell suspensions were maintained in a water bath at 37 °C with mild stirring and were steadily acidified with HCl (0.1; 0.5 and 1.0 N), so that the pH dropped gradually over time from pH 5 (at time 0) to 2.5 or 2.2 (depending on the assay) in a period of 90 minutes (see Figure 1). The pH was monitored with a pH meter (Orion 3 Star, Thermo Scientific; Beverly, MA, USA). Cell counts were carried out on MRS agar at the beginning (time 0) and after 60, 70, 75, 80, 85 and 90 min of simulated gastric digestion. Plates were incubated at 37 °C for 48 h.

Determination of RSGD along the growth curve

The growth curve of *L. casei* NAD was determined in MRS broth (absorbance at 560 nm and log CFU/mL plotted against time). *L. casei* NAD was harvested at 3 different points along its growth curve (early exponential growth phase (4 h), late exponential growth phase (12 h) and stationary growth phase (14 h), and RSGD was determined. A volume of 20 mL of each sample was centrifuged (4.000 × *g*, 10 min, 4 °C), washed twice with PBS and resuspended in 20 mL of RSM (10% w/v) (pH 6.5). The RSGD (final pH 2.2) was determined as described above.

Effect of the food matrix on RSGD

To evaluate the effect of the suspension medium on RSGD, a frozen culture of *L. casei* NAD was thawed and resuspended into 0.1% (w/v) peptone water (Britania, Buenos Aires, Argentina) or fermented milk (see below for details of fermented milk production). A given volume of the cell suspension was used to determine the RSGD (final pH 2.5) as described above.

Determination of RSGD during the refrigerated storage

An overnight culture of *L. casei* NAD was centrifuged (4.000 × *g*, 10 min, 4 °C), washed twice with PBS solution and resuspended in RSM (10% w/v) pH 6.5 or in RSM (10% w/v) acidified (with lactic acid, Sigma, St. Louis, MO, USA) to pH 4.5. Cell suspensions were aliquoted and stored at 5 °C or 12 °C during 30 days. The RSGD (final pH 2.2) was determined at 0, 15 and 30 days of storage.

Effect of inoculation time and refrigerated storage in fermented milk on RSGD

The influence of the inoculation time (addition of the probiotic before or after milk fermentation) and the subsequent refrigerated storage on RSGD was studied. A Yoghurt starter composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (cocci/lactobacilli rate 10:1) (Yo-Flex 700, type DVS, Chr. Hansen, Buenos Aires, Argentina) was used for fermented milk production according to the manufacturer's instructions. RSM 12% (w/v) was fermented at 43 °C until pH 4.5 was reached (approx. 5.5 h). A frozen concentrated culture of *L. casei* NAD was inoculated before or after fermentation with the Yoghurt starter, to an initial concentration of ca. 7 log orders. Both

© 2014 Society of Dairy Technology

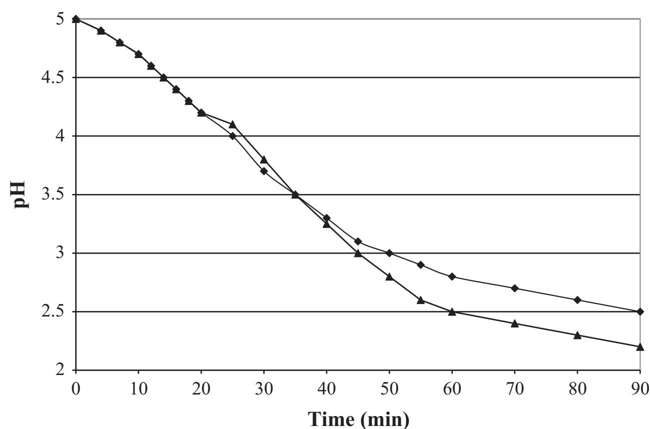

Figure 1 Acidification kinetics [final pH 2.2 (▲), final pH 2.5 (♦)] used to determine the resistance to simulated gastric digestion.

fermented milks, named IBF (inoculated before fermentation) and IAF (inoculated after fermentation), were aliquoted and kept at 5 °C and 12 °C for 30 days. The RSGD (final pH 2.5) was determined at the beginning and after 30 days of storage. When *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were present, MRS-LP (Vinderola *et al.* 2011a) agar was used for selective enumeration of *L. casei*.

Comparison of the resistance to simulated and real gastric fluid

Samples (5–8 mL) of human gastric fluid of 10 healthy volunteers (without endoscopic evidences of ulcers or other stomach disorders) were provided by courtesy of Dr. Marozzi and Dr. Nepote of the Instituto del Diagnóstico (Santa Fe, Argentina). Samples presenting a pH higher than 3.0 were discarded. The remaining five samples (pH values: 1.41; 1.92; 1.71; 2.46; 2.29) were pooled, centrifuged (4.000 × *g*, 15 min, 4 °C), filtered (0.45 μm, Millipore filter) and kept at −70 °C. The final pH of the pooled sample was 1.68.

Overnight cultures of *L. acidophilus* DRU, *L. casei* NAD, *L. paracasei* A13 and *L. casei* BIO were centrifuged (4.000 × *g*, 10 min, 4 °C) and washed twice with saline solution (0.85% (w/v) NaCl). Cells were harvested by centrifugation (4.000 × *g*, 10 min, 4 °C) and resuspended in simulated gastric fluid (SGF) (0.3% (w/v) pepsin, 0.5% (w/v) NaCl, pH 1.68 adjusted with HCl) or human gastric fluid (HGF). Cell suspensions were incubated on a water bath at 37 °C for 30 min. Cell counts were carried out on MRS agar, at the beginning (time 0) and after 10, 20 and 30 min of incubation. Plates were incubated at 37 °C for 48 h in aerobiosis.

Statistical analysis

All experiments were replicated at least twice on different days. Data were analysed using the one-way ANOVA procedure of SPSS software (SPSS Inc., Chicago, IL, USA). Data were considered significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

In this work, a bibliographic search was conducted on the conditions used by various researchers to *in vitro* study the tolerance of probiotic candidates to gastric or gastrointestinal digestion. Those conditions are summarised in Table 1. Except for temperature, parameters such as digestion time, pH and ingredients used to mimic gastric or intestinal fluid were found to significantly vary among reports. The majority of approaches used a constant pH exposure, while few reports explored the use of a gradual drop in pH from food pH to gastric pH (Mozzi *et al.* 2009; Kheadr *et al.* 2010), as it happens *in vivo*.

RSGD along the growth curve

Probiotic bacteria encounter various stressful conditions during biomass production and conservation, formulation into foods, food storage and gastrointestinal transit. During growth in a fermenter, several factors, such as culture pH, medium composition, harvesting time and gas atmosphere, can potentially affect the cell physiology in a way that may contribute to stability, for example a lower pH during growth can improve the viability on freeze-drying. Cells in the stationary growth phase are generally more tolerant to downstream stressful conditions than actively growing cells, maybe due to a more general stress response (Saarela *et al.* 2009).

In this study, the RSGD of *L. casei* NAD was seen to increase along the growth curve, being higher for cultures harvested late in the stationary growth phase (Figure 2). It is important to note that no significant differences ($P > 0.05$) were observed in cell counts between cultures harvested at early and at late stationary growth phase (data not shown). When the culture was harvested late in the stationary phase, viable cells were found after 90 min of simulated gastric digestion. However, no viable cells were found after 75 and 85 min of digestion for cultures harvested at early or late exponential growth phase, respectively. No differences in cell viability were observed by minute 60 of simulated digestion (pH was ca. 2.5 by that time), with viable cell counts being higher than 8.3 log orders. After 70 min of simulated digestion (pH was ca. 2.4), there was a difference in acid tolerance which depended on the culture harvesting time. A cell death of ca. 4, 2 and 0.9 log orders was observed for cells harvested at early, late exponential and stationary phase, respectively. These results agree with others found in the literature, which reported higher viability and functionality (measured as acid resistance) of *Lactobacillus* strains harvested at stationary phase than those harvested at exponential phase. This fact could be attributed to the induction of a general stress response by exposure to

© 2014 Society of Dairy Technology

Table 1 Ingredients and conditions used to assess the gastric or gastrointestinal resistance of probiotic bacteria to simulated digestion

Ingredients used for simulated gastrointestinal solution	*pH*	*Incubation*		*Reference*
		Temperature (°C)	*Time (min)*	
Pepsin, NaCl	2.0–2.5	37	180	Bao *et al.* 2010
Pepsin, NaCl, lipase	1.4–1.9	37	120	Buriti *et al.* 2010
Pepsin, Glucose, NaCl, KH_2PO_4, $CaCl_2$, KCl, porcine bile, lysozyme	1.85	37	30	Casey *et al.* 2004
Pepsin, NaCl	2.0	37	180	Charteris *et al.* 1998
Pepsin, MRS	2.5	37	120	Cho *et al.* 2009
Pepsin, NaCl, pancreatin, bile salts	2.0	37	120	Collado and Sanz 2007
Pepsin, Glucose, NaCl, KH_2PO_4, $CaCl_2$, KCl, porcine bile, lysozyme	2.0	37	90	Corcoran *et al.* 2005
Pepsin, NaCl, KCl, $NaHCO_3$	2.0–3.0	37	180	De Angelis *et al.* 2006
Pepsin, NaCl	3.2	37	60	Doleyres *et al.* 2004
Pepsin	2.0	37	60	Duc *et al.* 2004
Pepsin	2.0	37	60	Fakhry *et al.* 2008
Pepsin, NaCl, KCl, $NaHCO_3$	2.0–3.0	–	180	Fernández *et al.* 2003
Phosphate-buffered saline	2.0–3.0	37	180	Guo *et al.* 2010
Pepsin, NaCl, KCl, $NaHCO_3$	2.0–3.0	37	180	Hacin *et al.* 2008
Peptone water	2.0–4.0	37	90–180	Huang *et al.* 2007
MRS	2.0–3.0	37	180–360	Hyronimus *et al.* 2000
Pepsin	1.5–3.0	37	90	Izquierdo *et al.* 2008
Pepsin, MRS, GAM broth	2.5–3.0	37	180	Jonganurakkun *et al.* 2008
Pepsin, MRS	2.0–3.2	37	180	Jung *et al.* 2008
Pepsin, lipase	5.5–1.8[a]	37	360	Kheadr *et al.* 2010
MRS broth	2.0–4.0	37	180	Lähteinen *et al.* 2010
Pepsine, glucose, NaCl, KH_2PO_4, $CaCl_2$, KCl, porcine bile, lysozyme	2.0	–	90	Lebeer *et al.* 2008
Pepsin, MRS	2.5	37	120	Lee *et al.* 2008
BHI	3.0	37	180	Marcináková *et al.* 2010
MRS	2.0–2.5	37	120	Mathara *et al.* 2008
Phosphate-buffered saline	3.0	37	60–120	Mättö *et al.* 2004
Pepsin, NaCl	2.5	37	180	Mota *et al.* 2006
Pepsin, KCl, NaCl, $CaCl_2$, KH_2PO_4, mucin	2.0–3.0[a]	37	120	Mozzi *et al.* 2009
MRS	3.0	37	180	Nishida *et al.* 2008
Pepsin, MRS	2.0	37	60–180	Oh *et al.* 2000
Pepsin, NaCl	2.0	37	30–60	Patel *et al.* 2010
Pepsin, glucose, NaCl, KH_2PO_4, $CaCl_2$, KCl, porcine bile, lysozyme	2.0	37	90	Perea Vélez *et al.* 2007
MRS	2.0–3.0	–	240	Ryan *et al.* 2008
H_3PO_4	2.0	37	120	Sharp *et al.* 2008
HCl	–	37	60–120	Stadler and Viernstein 2003
Pepsin, glucose, NaCl, KH_2PO_4, $CaCl_2$, KCl, porcine bile, lysozyme	2.5	37	120	Sung *et al.* 2010
Phosphate-buffered saline	2.0–3.2	37	180	Tsai *et al.* 2004
Citrate buffer	3.0	37	300	Verdenelli *et al.* 2009
Pepsin, NaCl	3.0	37	60	Vinderola *et al.* 2007
Pepsin, saline solution, human gastric fluid	2.0	37	90	Zanoni *et al.* 2008

–, Data not provided. [a]Gradual drop of pH.

lactic acid (van de Guchte *et al.* 2002). Lorca and Font de Valdez (1999) described the effects of the physiological age of a *L. acidophilus* strain on the development of cross-resistance to various kinds of environmental stresses. These authors observed different degrees of resistance to acid exposure, freezing and oxidative stress in cultures at 37 °C, depending on the physiological age of the cells. Moreover, they reported the involvement of different sets of proteins in the cell adaptation process to environmental changes. According to their results, those proteins synthesised during starvation would be essential for acid resistance of the strain.

Effect of the food matrix on RSGD

Food, and fermented milks in particular, is the most widespread delivery system for probiotic bacteria and can, at

© 2014 Society of Dairy Technology

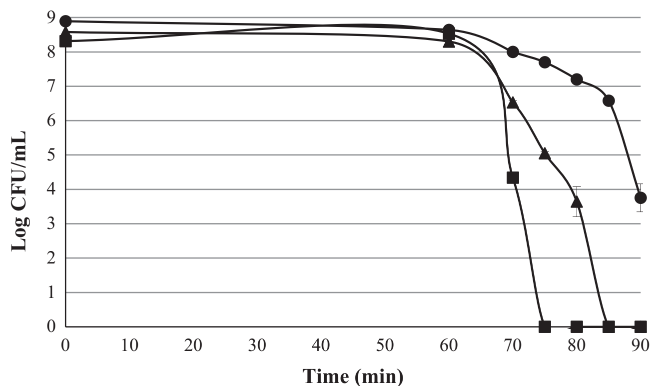

Figure 2 Cell viability of *Lactobacillus casei* NAD during the simulated gastric digestion of cell harvested at early (■) and late (▲) exponential growth phase and stationary growth phase (●).

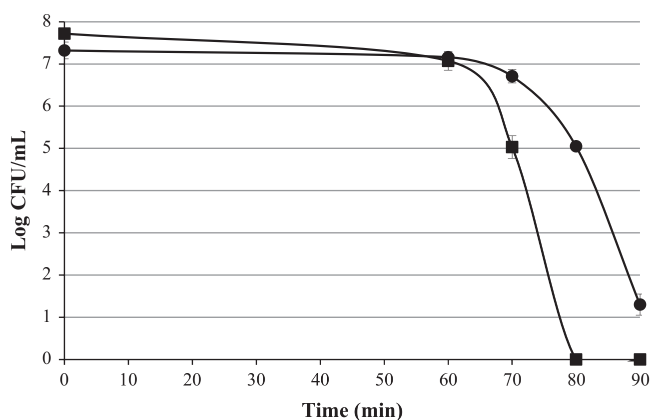

Figure 3 Cell viability of *Lactobacillus casei* NAD during the simulated gastric digestion of cultures suspended in (■) peptone water or in fermented milk (●).

least partially, protect them from acidic conditions (Wang *et al.* 2009). Fat and protein content, type of proteins, sugars, pH and certain food ingredients may affect their performance in this complex matrix, modifying their functionality and efficacy (Vinderola *et al.* 2011b). Figure 3 shows the *in vitro* RSGD of *L. casei* NAD resuspended in 0.1% (w/v) peptone water or fermented milk. The fermented milk matrix gave the strain a higher capacity to withstand the acidic conditions than peptone water, this may be due to the higher buffering capacity of the former, which decreases the effect of strong acids (Conway *et al.* 1987; Champagne and Gardner 2005; Ross *et al.* 2005). After 80 min of simulated digestion, no viable cells were detected when the strain was suspended in peptone water, whereas a decrease in cell viability of only 2.3 log orders was observed when using fermented milk. Similar results were reported by Wang *et al.* (2009). In this case, the survival rates of freshly prepared cultures of *L. casei* Zhang in simulated gastric juice at pH 2.0 and 2.5 were 31 and 69%, respectively, and the delivery of *L. casei* Zhang through fermented soymilk and bovine milk significantly improved the viability of the strain in simulated gastric transit. Charteris *et al.* (1998) reported a complete tolerance of a strain of *L. casei* and *B. infantis* to simulated gastric transit when adding sodium caseinate, whey protein isolate and a combination thereof.

Effect of refrigerated storage on RSGD

Subjection of microbial cells to a mild stress could induce resistance to a subsequent lethal challenge under the same stress condition. Moreover, exposure to sublethal levels of one stress condition can render cells resistant to other stress conditions. This becomes relevant in environments where micro-organisms can be exposed to a combination of stressing conditions, such as the human gastrointestinal tract (Burns *et al.* 2008). The incorporation of probiotic bacteria into fermented dairy products implies the necessity to maintain viable cells from production to consumption, where the cold chain plays a significant role for the maintenance of viability (Vinderola *et al.* 2011a). As previously shown by various authors (Brashears and Gilliland 1995; Nighswonger *et al.* 1996; Reilly and Gilliland 1999), cold storage can modify the functionality of probiotic bacteria. Accordingly, in this part of the study, we aimed at determining whether the cold storage of *L. casei* NAD in control (pH 6.5) or acidified RSM (pH 4.5) during 30 days at 5 °C and 12 °C may affect its RSGD. After analysing the results (Figure 4), *L. casei* NAD showed changes in both its viability and RSGD. The highest increases in both parameters were observed when the strain was stored for 30 days at 12 °C in control RSM (pH 6.5). Instead, the storage at 5 °C did not modify either the viability or the profile of RSGD, irrespective of the milk pH. However, a different behaviour was found when *L. casei* NAD was stored at 12 °C (mainly at pH 6.5). In this case, there was a positive effect (enhancement) on the RSGD and the strain was able to grow ca. 1 log order (12 °C, pH 6.5). Vinderola *et al.* (2011a) studied the *in vitro* RSGD of different *L. casei* strains in various commercial samples of fermented milks during refrigerated storage for 20 days at 5 °C and 12 °C. As in our study, in that case *L. casei* was able to grow during refrigerated storage. By day 20 at 12 °C, a temperature commonly found in retail display cabinets in supermarkets, cell counts were significantly higher (0.5–1 log orders) in certain commercial samples, compared to counts performed in samples immediately after their arrival to the laboratory. Moreover, the growth of *L. paracasei* in a commercial probiotic cheese held at 12 °C for 60 days had been previously reported (Vinderola *et al.* 2009). Champagne and Gardner (2008) compared the resistance of 4 *Lactobacillus* strains, as fresh cultures or after refrigerated storage, to simulated gastrointestinal

© 2014 Society of Dairy Technology

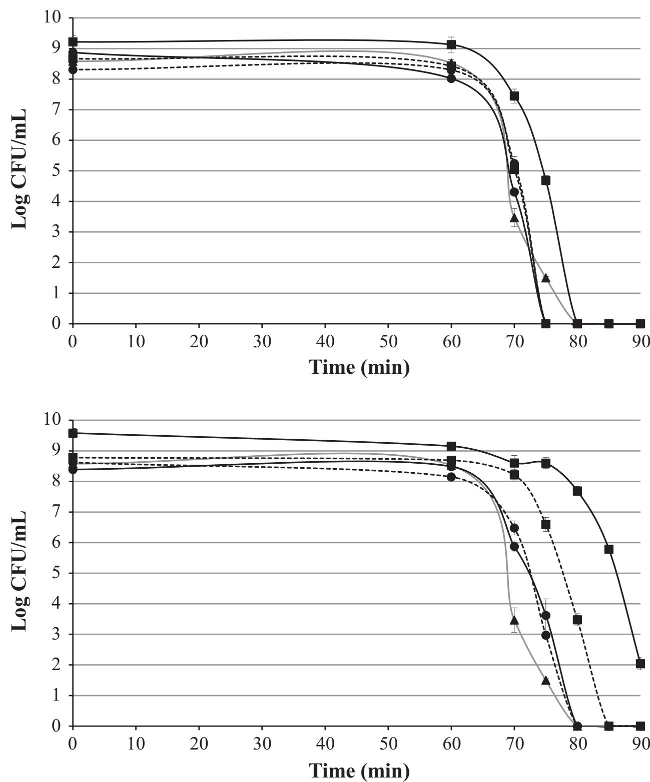

Figure 4 Cell viability of *Lactobacillus casei* NAD during the simulated gastric digestion in RSM (pH 6.5) (solid lines) or in RSM acidified to pH 4.5 (dashed lines), at time zero (▲) or after storage at 5 °C (●) and 12 °C (■) for 15 (above) or 30 (below) days.

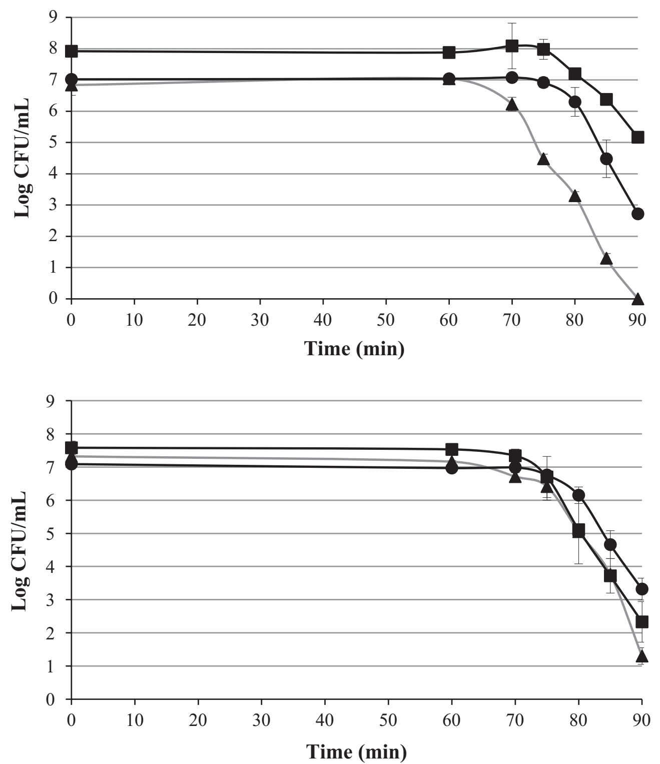

Figure 5 Cell viability of *Lactobacillus casei* NAD during the simulated gastric digestion of cultures inoculated in fermented milks before (above) or after (below) milk fermentation. RSGD was assessed in cultures immediately after inoculation (time zero: ▲) or after 30 days of storage of fermented milks at 5 °C (●) and 12 °C (■).

conditions. These authors found strong viability losses after incubation for 2 h (37 °C, pH 2.0). Furthermore, the cultures that were tested after having been stored for 35 days at 4 °C in the fruit drink had higher viability losses (ca. 1.2 log orders) than the fresh ones (harvested during stationary phase). These authors also concluded that a short-time exposure of the cultures to acid at 37 °C (during growth of fresh cultures) provides better protection to subsequent gastrointestinal conditions than does an extended low-temperature storage period. These controversial results indicate that companies wishing to develop new products need to carry out tests with the strains they have chosen in the specific food matrix (Champagne and Gardner 2008).

Effect of the inoculation time and the refrigerated storage on RSGD

Although many works reported the interaction between strains of starter cultures and probiotic bacteria (Shah 2000), no studies were found comparing the functionality of probiotics inoculated in fermented milks or yoghurts at different times during the fermentation process. The RSGD of *L. casei* NAD was lower when the strain was added together with the starter culture (before milk fermentation) than when it was inoculated after milk fermentation (Figure 5). This could indicate that the fermentation process had a negative impact on the strain viability during gastric digestion. After refrigerated storage for 30 days, the probiotic RSGD was higher than that of the initial time, irrespective of the time of inoculation. When *L. casei* NAD and the starter culture were inoculated together (IBF), after 30 days of storage at 12 °C, a cell death of only 2.8 log orders was found after 90 min of simulated digestion, while no viable cells were found by this time when the RSGD was evaluated immediately after fermentation (time 0). This behaviour was not observed when the probiotic was inoculated after the fermentation process (IAF). To the best of our knowledge, this is the first report showing changes in resistance to simulated gastric transit related to a technological variable (time of inoculation: before milk fermentation or once milk fermentation is completed). Even not unveiled yet, the mechanisms underlying these different behaviours might be influenced by different factors such us temperature (high temperature of exposure when cells are added in IBF products compared to IAF products) and exposure to products derived from lactic acid fermentation

© 2014 Society of Dairy Technology

(lactic acid or other organic acids or bacteriocin-like compounds).

In this work, a final pH of 2.2 or 2.5 was used along the different experiments of simulated dynamic gastric digestion. This was due to the different sensitivity of probiotic cells to simulated gastric digestion in those experiments. Taking into account that this is a simplified simulation of a much more complex process, in some cases we decided to use milder conditions (final pH 2.5 instead of 2.2) in order to find viable cells of lactobacilli by the end of the simulated gastric digestion, which allowed us to compare the different technological conditions assessed (moment/time of inoculation, storage, food matrix).

Comparison of the resistance to simulated and real gastric fluid

The low pH of the stomach, together with the proteolytic activity of the digestive enzymes, is the first and one of the most important challenges to be overcome by a probiotic culture on its passage through the gastrointestinal tract. In most studies, authors employ simulated gastric solutions but just a few of them evaluate the gastric resistance using human gastric fluid (Table 1), possibly due to the difficulty of obtaining these kind of samples. The resistance of some probiotic strains was assessed after 10, 20 and 30 min of incubation in simulated gastric fluid (SGF) (Table 2) and in human gastric fluid (HGF). The viability of *L. acidophilus* DRU did not change when using SGF (after 30 min of incubation a negligible cell death of 0.3 log orders was observed); however, no viable cells were found when cells were resuspended in HGF, even after 10 min of incubation (data not shown). This fact indicates that other factors, besides the SGF pH value, affect the gastric resistance of a micro-organism. On the contrary, *L. casei* strains showed high sensitivity to SGF. In fact, for *L. casei* NAD, a cell death of 5.8 log orders was found after 10 min of incubation and no viable cells were found later on. Similar to *L. acidophilus* DRU, *L. casei* NAD was highly sensitive when incubated in human gastric fluid (data not shown). Zanoni *et al.* (2008) compared the resistance of *L. plantarum*, *S. thermophilus* and *B. lactis* in human gastric juice, simulated gastric juice and bile and found that all the strains exhibited complete loss of viability after 90 min in human gastric juice (pH 1.8). Even though a high difference in cell viability was observed when SGF or HGF was used, other factors should be considered when evaluating gastric tolerance and formulating simulated gastric solutions; namely, a more sophisticated *in vitro* approach is certainly still needed.

The gastric pH of healthy people is normally around 1.8 but can increase to values ranging from 3 to 5 after eating (Zanoni *et al.* 2008). Individual differences, meal composition and gastric emptying time are other variables that could have an effect on gastric tolerance. The determination of the gastric resistance using HGF or SGF to an extremely low and constant pH during a long time may be underestimating the real gastric resistance of a micro-organism. Probiotic bacteria are normally delivered in a food matrix so the pH and the time at which they are exposed in the stomach may not be the same for every single cell because, while some cells are entering the stomach and pH is starting to drop, other cells might be already inside it and others might also be leaving it towards the duodenum. In this regard, Berrada *et al.* (1991) reported that in humans, gastric emptying is a dynamic process whereby after 40 min of digestion, approximately 50% of the ingested fermented milk already left the stomach. In our acidification curve, by min 40, pH was still above 3.2. Then, conditions used in *in vitro* models for the estimation of resistance to gastric digestion (constant and low pH values) might be still too harsh and then might be underestimating the present tolerance to the passage through the stomach. Berrada *et al.* (1991) also demonstrated that the *in vitro* relative tolerance between strains is a good estimation of the *in vivo* tolerance.

Table 2 Cell counts (log CFU/mL ± SD) of *Lactobacillus* strains in simulated gastric solution (pH 1.68)

	Time of simulated digestion (min)			
Strain	*0*	*10*	*20*	*30*
L. acidophilus DRU	8.09[a]	8.20[a]	8.12[a]	7.79[a]
L. casei NAD	8.16[a]	2.34[b]	<1	<1
L. casei BIO	8.07[a]	3.93[b]	1.30[c]	<1
L. paracasei A13	8.12[a]	3.35[b]	1.48[c]	<1

[a,b,c]Values in rows with different superscript are significantly different ($P < 0.05$).

CONCLUSIONS

These results indicate that the variables assessed affected both the viability and the functionality of the strains studied and demonstrate that viability does not always correlate directly with functionality. This is an important fact because both parameters are necessary for probiotics to exert a benefit on health. Moreover, the diversity of available protocols for assessment of RSGD suggests the need of a standardised one for monitoring the effect of diverse processing and storage conditions on the gastric resistance of probiotic bacteria and for a better comparison of probiotic candidates assessed around the world.

ACKNOWLEDGEMENTS

This work was supported by the project 'Nuevas estrategias para aumentar la funcionalidad de alimentos y cepas probióticas'. PICT Bicentenario 2010 N° 0161. ANPCyT, 2011–2013.

© 2014 Society of Dairy Technology

REFERENCES

Bao Y, Zhang Y, Zhang Y, Liu Y, Wang S, Dong X, Wang Y and Zhang H (2010) Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control* **21** 695–701.

Berrada N, Lemeland J F, Laroche G, Thouvenot P and Piaia M (1991) *Bifidobacterium* from fermented milks: survival during gastric transit. *Journal of Dairy Science* **74** 409–413.

Brashears M M and Gilliland S E (1995) Survival during frozen and subsequent refrigerated storage of *Lactobacillus acidophilus* cells as influenced by the growth phase. *Journal of Dairy Science* **78** 2326–2335.

Buriti F C A, Castro I A and Saad S M I (2010) Viability of *Lactobacillus acidophilus* in synbiotic guava mousses and its survival under *in vitro* simulated gastrointestinal conditions. *International Journal of Food Microbiology* **137** 121–129.

Burns P, Vinderola G, Binetti A, Quiberoni A, de los Reyes Gavilán C G and Reinheimer J (2008) Bile resistance derivatives obtained from non-intestinal dairy lactobacilli. *International Dairy Journal* **18** 377–385.

Casey P G, Casey G D, Gardiner G E, Tangney M, Stanton C, Ross R P, Hill C and Fitzgerald G F (2004) Isolation and characterization of anti-*Salmonella* lactic acid bacteria from the porcine gastrointestinal tract. *Letters in Applied Microbiology* **39** 431–438.

Champagne C P and Gardner N J (2005) Challenges in the addition of probiotic cultures to foods. *Critical Reviews in Food Science and Nutrition* **45** 61–84.

Champagne C P and Gardner N J (2008) Effect of storage in a fruit drink on subsequent survival of probiotic lactobacilli to gastro-intestinal stresses. *Food Research International* **41** 539–543.

Charteris W P, Kelly P M, Morelli L and Collins J K (1998) Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology* **84** 759–768.

Cho I J, Lee N K and Hahm Y T (2009) Characterization of *Lactobacillus* spp. isolated from the feces of breast-feeding piglets. *Journal of Bioscience and Bioengineering* **108** 194–198.

Collado M C and Sanz Y (2007) Induction of acid resistance in *Bifidobacterium*: a mechanism for improving desirable traits of potentially probiotic strains. *Journal of Applied Microbiology* **103** 1147–1157.

Conway P L, Gorbach S L and Goldin B R (1987) Survival of Lactic Acid Bacteria in the human stomach and adhesion to intestinal cells. *Journal of Dairy Science* **70** 1–12.

Corcoran B M, Stanton G, Fitgerald C F and Ross R P (2005) Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology* **71** 3060–3067.

De Angelis M, Siragusa S, Berloco M, Caputo L, Settanni L, Alfonsi G, Amerio M, Grandi A, Ragni A and Gobbetti M (2006) Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. *Research in Microbiology* **157** 792–801.

Doleyres Y, Fliss I and Lacroix C (2004) Increased stress tolerance of *Bifidobacterium longum* and *Lactococcus lactis* produced during continuous mixed-strain immobilized-cell fermentation. *Journal of Applied Microbiology* **97** 527–539.

Duc L H, Hong H A, Barbosa T M, Henriques A O and Cutting S M (2004) Characterization of *Bacillus* probiotics available for human use. *Applied and Environmental Microbiology* **70** 2161–2171.

Fakhry S, Sorrentini I, Ricca E, De Felice M and Baccigalupi L (2008) Characterization of spore forming bacilli isolated from the human gastrointestinal tract. *Journal of Applied Microbiology* **105** 2178–2186.

FAO/WHO (Food and Agriculture Organization/World Health Organization) (2002) Guidelines for the Evaluation of Probiotics in Food. Londres, Ontario, Canadá. URL http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf. Accessed 7/7/2013.

Fernández M F, Boris S and Barbés C (2003) Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *Journal of Applied Microbiology* **94** 449–455.

van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich S D and Maguin E (2002) Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek* **82** 187–216.

Guo X H, Kim J M, Nam H M, Park S Y and Kim J M (2010) Screening lactic acid bacteria from swine origins for multistrain probiotics based on *in vitro* functional properties. *Anaerobe* **16** 321–326.

Hacin B, Rogelj I and Matijasic B B (2008) *Lactobacillus* isolates from weaned piglets' mucosa with inhibitory activity against common porcine pathogens. *Folia Microbiology* **53** 569–576.

Huang H Y, Huang S Y, Chen P Y, King V A E, Lin Y P and Tsen J H (2007) Basic characteristics of *Sporolactobacillus inulinus* BCRC 14647 for potential probiotic properties. *Current Microbiology* **54** 396–404.

Hyronimus B, Le Marrec C, Hadj Sassi A and Deschamps A (2000) Acid and bile tolerance of spore-forming lactic acid bacteria. *International Journal of Food Microbiology* **61** 193–197.

Izquierdo E, Medina M, Ennahar S, Marchioni E and Sanz Y (2008) Resistance to simulated gastrointestinal conditions and adhesion to mucus as probiotic criteria for *Bifidobacterium longum* strains. *Current Microbiology* **56** 613–618.

Jonganurakkun B, Wang Q, Xu S H, Tada Y, Minamida K, Yasokawa D, Sugi M, Hara H and Asano K (2008) *Pediococcus pentosaceus* NB-17 for probiotic use. *Journal of Bioscience and Bioengineering* **106** 69–73.

Jung S W, Kim W J, Lee K G, Kim C W and Noh W S (2008) Fermentation characteristics of exopolysaccharide-producing Lactic Acid Bacteria from sourdough and assessment of the isolates for industrial potential. *Journal of Microbiology and Biotechnology* **18** 1266–1273.

Kheadr E, Zihler A, Dabour N, Lacroix C, Le Blay G and Fliss I (2010) Study of the physicochemical and biological stability of pediocin PA-1 in the upper gastrointestinal tract conditions using a dynamic *in vitro* model. *Journal of Applied Microbiology* **109** 54–64.

Lähteinen T, Malinen E, Koort J M K *et al.* (2010) Probiotic properties of *Lactobacillus* isolates originating from porcine intestine and feces. *Anaerobe* **16** 293–300.

Lebeer S, Claes I J J, Verhoeven T L A, Shen C, Lambrichts I, Ceuppens J L, Vanderleyden J and De Keersmaecker S C J (2008) Impact of *luxS* and suppressor mutations on the gastrointestinal transit of *Lactobacillus rhamnosus* GG. *Applied and Environmental Microbiology* **74** 4711–4718.

Lee N K, Yun C W, Kim S W, Chang H Y, Kang C W and Paik H D (2008) Screening of lactobacilli derived from chicken feces and

© 2014 Society of Dairy Technology

partial characterization of *Lactobacillus acidophilus* A12 as animal probiotics. *Journal of Microbiology and Biotechnology* **18** 338–342.

Lorca G L and Font de Valdez G (1999) The effect of suboptimal growth temperature and growth phase on resistance of *Lactobacillus acidophilus* to environmental stress. *Cryobiology* **39** 144–149.

Mainville I, Arcand Y and Farnworth E R (2005) A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *International Journal of Food Microbiology* **99** 287–296.

Marcináková M, Klingberg T D, Laukovaá A and Budde B B (2010) The effect of pH, bile and calcium on the adhesion ability of probiotic enterococci of animal origin to the porcine jejunal epithelial cell line IPEC-J2. *Anaerobe* **16** 120–124.

Marteau P, Minekus M, Havenaar R and Huis in't Veld J H J (1997) Survival of Lactic Acid Bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *Journal of Dairy Science* **80** 1031–1037.

Mathara J M, Schillinger U, Guigas C, Franz C, Kutima P M, Mbugua S K, Shin H K and Holzapfel W H (2008) Functional characteristics of *Lactobacillus* spp. from traditional Maasai fermented milk products in Kenya. *International Journal of Food Microbiology* **126** 57–64.

Mättö J, Malinen E, Suihko M L, Alander M, Palva A and Saarela M (2004) Genetic heterogeneity and functional properties of intestinal bifidobacterias. *Journal of Applied Microbiology* **97** 459–470.

Morelli L (2007) *In vitro* assessment of probiotic bacteria: from survival to functionality. *International Dairy Journal* **17** 1278–1283.

Mota R M, Moreira J L S, Souza M R, Horta M F, Teixeira S M R, Neumann E, Nicoli J R and Nunes A C (2006) Genetic transformation of novel isolates of chicken *Lactobacillus* bearing probiotic features for expression of heterologous proteins: a tool to develop live oral vaccines. *BMC Biotechnology* **6** 2.

Mozzi F, Gerbino E, Font de Valdez G and Torino M I (2009) Functionality of exopolysaccharides produced by Lactic Acid Bacteria in an *in vitro* gastric system. *Journal of Applied Microbiology* **107** 56–64.

Nighswonger B D, Brashears M M and Gilliland S E (1996) Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk products during refrigerated storage. *Journal of Dairy Science* **79** 212–219.

Nishida S, Michinaka A, Nakashima K, Iino H and Fujii T (2008) Evaluation of the probiotic potential of *Lactobacillus paracasei* KW3110 based on *in vitro* tests and oral administration tests to healthy adults. *Journal of General and Applied Microbiology* **54** 267–276.

Oh S, Kim S H and Worobo R W (2000) Characterization and purification of a bacteriocin produced by a potential probiotic culture, *Lactobacillus acidophilus* 30SC. *Journal of Dairy Science* **83** 2747–2752.

Patel A K, Ahire J J, Pawar S P, Chaudhari B L, Shouche Y S and Chincholkar S B (2010) Evaluation of probiotic characteristics of siderophoregenic *Bacillus* spp. isolated from dairy waste. *Applied Biochemistry and Biotechnology* **160** 140–155.

Perea Vélez M, Hermans K, Verhoeven T L A, Lebeer S E, Vanderleyden J and De Keersmaecker S C J (2007) Identification and characterization of starter Lactic Acid Bacteria and probiotics from Columbian dairy products. *Journal of Applied Microbiology* **103** 666–674.

Reilly S S and Gilliland S E (1999) *Bifidobacterium longum* survival during frozen and refrigerated storage as related to pH during growth. *Journal of Food Science* **64** 714–718.

Ross R P, Desmond C, Fitzgerald G F and Stanton C (2005) Overcoming the technological hurdles in the development of probiotic foods. *Journal of Applied Microbiology* **98** 1410–1417.

Ryan K A, Jayaraman T, Daly P, Canchaya C, Curran S, Fang F, Quigley E M and O'Toole P W (2008) Isolation of lactobacilli with probiotic properties from the human stomach. *Letters in Applied Microbiology* **47** 269–274.

Saarela M, Virkajarvi I, Alakomi H L, Sigvart-Mattila P and Mättö J (2006) Stability and functionality of freeze-dried probiotic *Bifidobacterium* cells during storage in juice and milk. *International Dairy Journal* **16** 1477–1482.

Saarela M H, Alakomi H L, Puhakka A and Mättö J (2009) Effect of the fermentation pH on the storage stability of *Lactobacillus rhamnosus* preparations and suitability *of in vitro* analyses of cell physiological functions to predict it. *Journal of Applied Microbiology* **106** 1204–1212.

Shah N P (2000) Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of Dairy Science* **83** 894–907.

Sharp M D, McMahon D J and Broadbent J R (2008) Comparative evaluation of yogurt and low-fat Cheddar cheese as delivery media for probiotic *Lactobacillus casei*. *Journal of Food Science* **73** 375–377.

Stadler M and Viernstein H (2003) Optimization of a formulation containing viable Lactic Acid Bacteria. *International Journal of Pharmaceutics* **256** 117–122.

Sung C, Kim B G, Kim S, Joo H S and Kim P I (2010) Probiotic potential of *Staphylococcus hominis* MBBL 2–9 as anti-*Staphylococcus aureus* agent isolated from the vaginal microbiota of a healthy woman. *Journal of Applied Microbiology* **108** 908–916.

Tsai C C, Huanga L F, Lina C C and Tsen H Y (2004) Antagonistic activity against *Helicobacter pylori* infection *in vitro* by a strain of *Enterococcus faecium* TM39. *International Journal of Food Microbiology* **96** 1–12.

Verdenelli M C, Ghelfi F, Silvi S, Orpianesi C, Cecchini C and Cresci A (2009) Probiotic properties of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from human faeces. *European Journal of Nutrition* **48** 355–363.

Vinderola G, Briggiler Marcó M, Guglielmotti D M, Perdigón G, Giraffa G, Reinheimer J and Quiberoni A (2007) Phage-resistant mutants of *Lactobacillus delbrueckii* may have functional properties that differ from those of parent strains. *International Journal of Food Microbiology* **116** 96–102.

Vinderola G, Molinari F, Prosello W, Ghiberto D and Reinheimer J (2009) Growth of *L. paracasei* A13 in Argentinean probiotic cheese. Impact on the characteristics of the product. *International Journal of Food Microbiology* **135** 171–174.

Vinderola G, Céspedes M, Mateolli D, Cardenas P, Lescano M, Aimaretti N and Reinheimer J (2011a) Changes in gastric resistance of *Lactobacillus casei* in flavoured commercial fermented milks during refrigerated storage. *International Journal of Dairy Technology* **64** 269–275.

Vinderola G, Binetti A, Burns P and Reinheimer J (2011b) Cell viability and functionality of probiotic bacteria in dairy products. *Frontiers in Microbiology* **2** 1–6.

 © 2014 Society of Dairy Technology

Vinderola G, Zacarías M F, Bockelmann W, Neve H, Reinheimer J and Heller K J (2012) Preservation of functionality of *Bifidobacterium animalis* subsp. *lactis* INL1 after incorporation of freeze-dried cells into different food matrices. *Food Microbiology* **30** 274–280.

Wang J, Guo Z, Zhang Q, Yan L, Chen W, Liu X-M and Zhang H P (2009) Fermentation characteristics and transit tolerance of probiotic *Lactobacillus casei* Zhang in soymilk and bovine milk during storage. *Journal of Dairy Science* **92** 2468–2476.

Zanoni S, Pompei A, Cordisco L, Amaretti A, Rossi M and Matteuzzi D (2008) Growth kinetics on oligo- and polysaccharides and promising features of three antioxidative potential probiotic strains. *Journal of Applied Microbiology* **105** 1266–1276.

© 2014 Society of Dairy Technology