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Short communication

Variability in the content of *Lactobacillus casei* in different commercial lots of fermented milks

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Introduction

Fermented dairy products containing living probiotic microorganisms have been largely proposed as a natural means to restore or to enhance gut health (Sánchez *et al*., 2009). When incorporating probiotic cultures into fermented dairy products, there is the need to maintain viable cells from production to consumption (Ross *et al*., 2005), to cover the expectancy of the consumer about a functional food. Many factors were pointed out as responsible for adequate viability of probiotics in fermented milks, such as the type and flavour of the product (Birollo *et al*., 2000; Saarela *et al*., 2006; Vinderola *et al*., 2011b), the chemical ingredients used for product manufacture (Vinderola *et al*., 2002a) and the possible interactions with the starters used for milk fermentation (Vinderola *et al*., 2002b). Although a certain degree of effectiveness is recognised to nonviable cells, cell viability is a prerequisite for cell functionality (Ouwehand & Salminen, 1998). At the same time, cell functionality is highly influenced by the food matrix components (Ranadheera *et al*., 2010). It was reported that the strain *L. rhamnosus* GG displayed different inhibitory capacity towards pathogens depending on the food matrix used as vehicle (Grzeskowiak *et al*., 2011). In general, the indicated shelf life of fermented milks is about 30–45 days (Tamime & Robinson, 2000), and then different commercial lots are made available to consumers along the year. Changes in some features of interest were reported for the same probiotic culture in fermented dairy products belonging to different commercial lots. For instance, different adhesion capacity to eukaryotic cells was reported for the same strain of *Lactobacillus acidophilus* isolated at different years from the same fermented milk (Tuomola *et al*., 2001). Authors concluded that changes in the adhesion capacity apparently occurred during industrial production. In a previous work, we reported variable contents of probiotic bacteria in a fresh cheese along 10 years of industrial production (Vinderola *et al*., 2009).

Some of the factors that drive consumer's willingness to buy a probiotic food are primary health concerns, consumers' familiarity with the concept of functional foods, the nature of the carrier product, the communication mode, packaging, brand, taste and price (Annunziata & Vecchio, 2013). However, one important factor that warrants the product functionality, and that cannot be perceived neither controlled by consumers, is the level of viable cells present at the moment of buying a probiotic food. From the perspective of the consumer, the adequate amount of viable cells present in probiotic product is a desirable attribute that they trust on, to obtain the expected health benefit. Additionally, an informed consumer might expect to obtain reasonably the same amount of active cultures whenever he gets the product from the display cabinet at the supermarket. In this work, we aimed at monitoring cell viability and resistance to simulated gastric digestion in four probiotic fermented milks available in the Argentinian market along nine different commercial lots sampled in a frame time of 15 months.

Materials and methods

Fermented milk samples

Fermented milk samples from nine different commercial lots (industrial batches) from brand A (flavours

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strawberry and vanilla) and brand B (flavours strawberry and multifruits) were purchased always in the same supermarket in the city of Rosario (Santa Fe, Argentina). Samples were acquired in the months of September, October and November 2012 and May, June, August, September, October and November 2013 (nine different commercial lots that were numbered successively from 1 to 9). The specific commercial lot code (a combination of letters and numbers) was registered to make sure samples corresponded to different industrial batches. Fermented milks were transported under refrigeration conditions at 5 °C to the laboratory in <30 min after buying and kept at 5 °C until analysed within the next 2 days. The information about commercial brand, identity of the samples and the manufacturer was kept undisclosed for confidentiality reasons and to avoid any conflict of interest with the kind of results obtained. Samples from the two commercial brands claimed to include one (different) probiotic strain of *Lactobacillus casei*. The identity of the probiotic strains in these products is periodically verified by molecular tools in our laboratory as part of a programme of quality control for the local dairy industries.

Enumeration of *Lactobacillus casei* in fermented milk samples and during simulated gastric digestion

Selective cell counts of *L. casei* were performed by plating serial dilutions of the fermented milks [in 0.1% (w/v) casein peptone; Microquim S.A., Santa Fe, Argentina] on MRS agar (Biokar, Beauvais, France) containing 0.2% (w/v) lithium chloride (Sigma, St. Louis, MO, USA) and 0.3% (w/v) sodium propionate (Sigma), according to Vinderola & Reinheimer (2000). Plates were incubated at 37 °C for 48 h in aerobiosis. Cell morphology was confirmed by light microscopy examination. For simulated gastric digestion, a volume (25 mL) of fermented milk was mixed with the same volume of a 'salive-gastric'-resembling 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 0.6% (w/v) porcine pepsine (Marteau *et al.*, 1997). A 1-mL sample was removed for cell counts immediately after mixture, and pH was quickly brought to 2.50 with 5 M and 0.1 M HCl. Samples were brought to 37 °C in a water bath and maintained for 90 min. Aliquots (1 mL) were taken every 30 min, and serial dilutions were plated for cell viability assessment.

Statistical analysis

Assays were performed, in independent duplicates on the same day (using two different samples bought on the same day too), by three different people. Data were analysed using one-way ANOVA, with the SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA). The differences between means were detected by the Tukey's multiple range test. Data were considered significantly different when $P < 0.05$.

Results and discussion

The level of viable cells of *L. casei* among commercial lots was very variable and ranged from 7.65 to 8.73, 7.85 to 8.76, 5.98 to 7.46 and 5.92 to 7.11 for the fermented milks of brand A (flavour strawberry and vanilla, Fig. 1) and brand B (flavour strawberry and multifruits, Fig. 2), respectively. This mean a difference of cell counts that ranged from 0.9 to 1.5 among commercial lots, depending on the product considered. In all cases, cell counts among commercial lots of the same product, at the moment of buying the product in the supermarket, were significantly different ($P = 0.000$). The content of viable probiotic bacteria is still an issue of debate, specially when trying to set regulatory policies for quality control. It is likely that the required amount of probiotics should be different among probiotic strains as functional effects are strain and dose dependant (Minelli & Benini, 2008; Fang *et al.*, 2009). However, there is an international trend to consider that fermented milks should contain at least 10^7–10^8 cfu mL^{-1} of viable cells of the probiotic strain (Champagne & Gardner, 2005). In general, positive health effects have been observed in human clinical studies with fermented milks containing these amounts of probiotics (Montrose & Floch, 2005). In this context, both products of the brand A contained, in all sampling points, amounts of *L. casei* commonly considered as effective to promote health, whereas for some lots of the products of the brand B, quantities of viable cells as low as 5.98 log orders were observed. Since the launch of fermented milks carrying probiotics into the market, scientific works about unsatisfactory counts of probiotic bacteria during cold storage of the products were reported in Australia (Micanel *et al.*, 1995; Shah *et al.*, 1995), Germany (Schillinger, 1999), Argentina (Vinderola *et al.*, 2000), Italy (Fasoli *et al.*, 2003), Spain (Gueimonde *et al.*, 2004) and France (Coeuret *et al.*, 2004), among other reports.

The resistance to simulated gastric digestion was also evaluated by exposure to the fermented milk to pH 2.5 in the presence of pepsine for 90 min at 37 °C. As already observed for counts of viable cells in the different products, cell decays after 30, 60 and 60 min of exposure to simulated gastric acidity were significantly different ($P = 0.000$) among commercial lots of all products assessed. Cell death (in log orders) due to simulated gastric digestion ranged from 0.1 to 2.2, 0.2 to 1.8, 0.5 to 2.5 and 0.6 to 2.5 for the fermented milks of brand A (flavour strawberry and vanilla, Fig. 1) and brand B (flavour strawberry and multi-

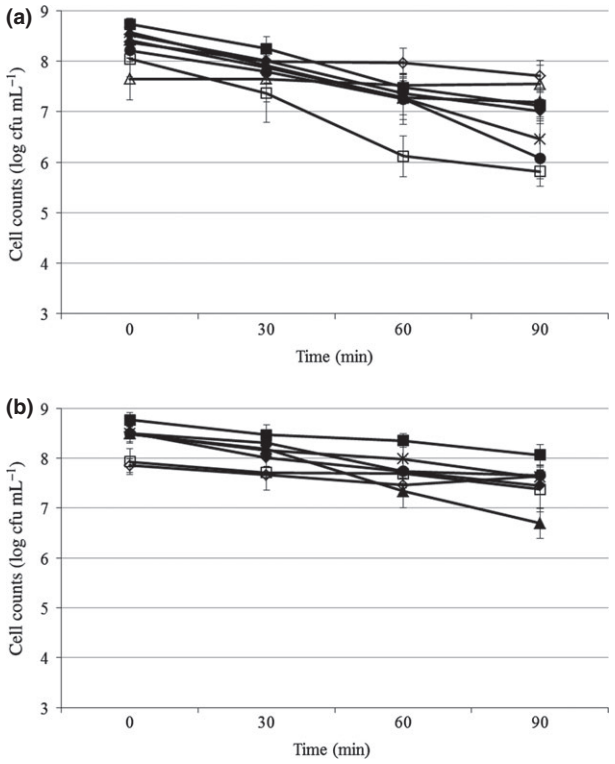

Figure 1 Cell counts of *Lactobacillus casei* during the simulated gastric digestion of fermented milks of the brand A-flavour strawberry (a) and vanilla (b). Commercial lots 1–8, symbols ♦, ■, ▲, ●, x, ◊, □ and Δ, respectively. Commercial lot 9 for flavour strawberry and commercial lots 8 and 9 for flavour vanilla were not assessed.

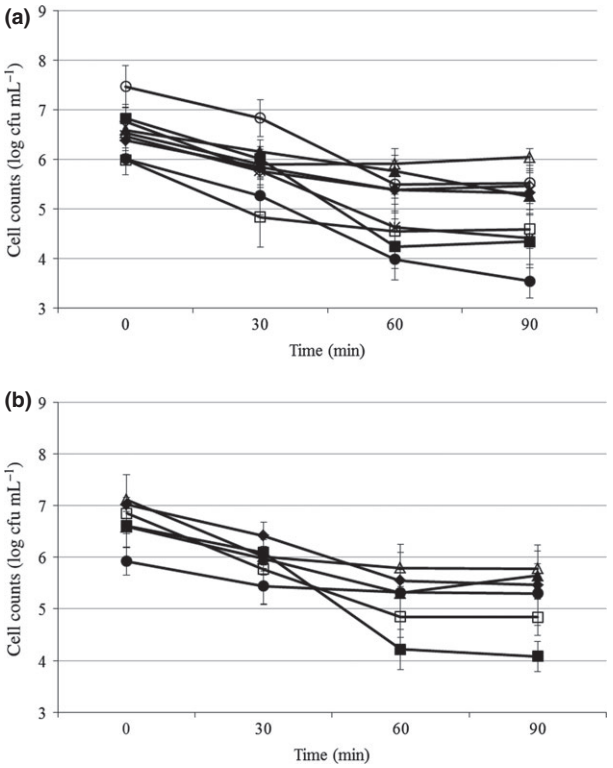

Figure 2 Cell counts of *Lactobacillus casei* during the simulated gastric digestion of fermented milks of the brand B-strawberry (a) and multifruits (b). Commercial lots 1–9, symbols ♦, ■, ▲, ●, x, ◊, □, Δ and ○, respectively. Commercial lots 5, 6 and 9 for flavour multifruits were not assessed.

fruits, Fig. 2). However, no significant differences ($P = 0.157$) were observed in the mean reduction in cell counts (ranging from 0.9 to 1.6 log orders) among the four products assessed (Fig. 3). We also compared initial cell counts and counts after 90 min of simulated gastric digestion for each commercial lot for each product. Significant reductions ($P = 0.000$) were observed after simulated gastric digestion for both products of the brand A and the product of flavour strawberry of the brand B. However, no significant differences ($0.883 < P < 0.999$) were observed for the 6 lots studied of the product brand B flavour multifruits. The latter was the product presenting the lowest initial counts among the four products assessed. In a previous work (Céspedes *et al.*, 2013), a fast lost in cell viability was observed for *L. casei* immediately after its addition to fruit juice, whereas another fraction of cells remained viable and without changes on its resistance to simulated gastric digestion along storage. These results reveal the presence of heterogenicity within the population of probiotic cells in relation to

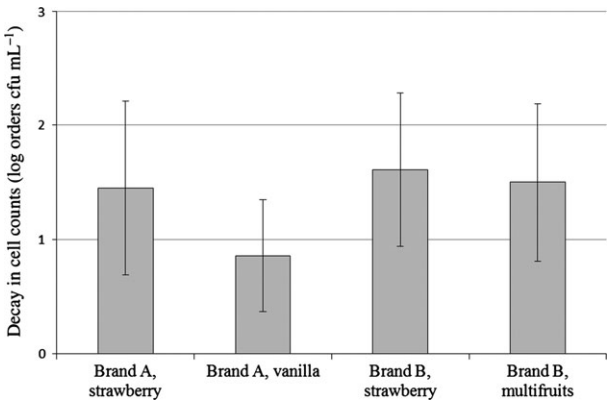

Figure 3 Reductions in cell counts of *Lactobacillus casei* after simulated gastric digestion in fermented milks of different commercial lots (brand A-strawberry: $n = 8$, brand A-vanilla: $n = 7$, brand B-strawberry: $n = 9$, brand B-multifruits: $n = 6$, n: number of commercial lots assessed for each brand flavour). Bars are the mean ± SD of the differences in cell counts at time 0 and after 90 min of simulated gastric digestion. Reductions in cell counts were not significantly different ($P = 0.157$).

the resistance to acidic conditions. Within an isogenic microbial population in a homogenous environment, individual bacteria can still exhibit differences in phenotype. Phenotypic heterogeneity can facilitate the survival of subpopulations under stress (Ingham *et al.*, 2008). In a previous work, we also reported that resistance to simulated gastric digestion changed during the shelf life of the products maintained at 5 °C, depending again on the brand and flavour considered (Vinderola *et al.*, 2011a). Many microbiological and technological factors were signalled as responsible for inducing changes in the functionality of probiotic bacteria in food (Ranadheera *et al.*, 2010) and in particular in fermented milks (Vinderola *et al.*, 2011b). In this work, the same strain exhibited different resistance to simulated gastric digestion in different food products. Grzeskowiak *et al.* (2011) reported that permanent changes in the antimicrobial capacity of *Lactobacillus* GG were verified after its passage through food and pharmaceutical products. The mechanisms that support the changes in resistance to simulated gastric resistance must be further studied; however, this report is the first on its kind to point out that differences in functionality might be observed for the same strain along different commercial lots of the same product. Results reported in this work have to be considered in order to guarantee a quality control system along the commercial lots and to stimulate specific experiments in order to understand the involved mechanisms.

Conclusions

Differences in cell counts of *L. casei* (from 0.9 to 1.5 log orders) were found in four different probiotic fermented milks along nine commercial lots monitored from June 2012 to November 2013. For certain products, the level of viable cells was below that recommended by the international consensus (10^7 cfu mL^{-1}) or considered as effective in human clinical trials. The instability in the content of probiotic bacteria in commercial products might still be an issue to be considered by food technologists and by the local authorities in charge of monitoring the quality of these kinds of products.

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