Performance in Nondairy Drinks of Probiotic *L. casei* Strains Usually Employed in Dairy Products

Mario Céspedes, Pamela Cárdenas, Martín Staffolani, María C. Ciappini, and Gabriel Vinderola

Abstract: The increase in vegetarianism as dietary habit and the increased allergy episodes against dairy proteins fuel the demand for probiotics in nondairy products. Lactose intolerance and the cholesterol content of dairy products can also be considered two additional reasons why some consumers are looking for probiotics in other foods. We aimed at determining cell viability in nondairy drinks and resistance to simulated gastric digestion of commercial probiotic lactobacilli commonly used in dairy products. *Lactobacillus casei* LC-01 and *L. casei* BGP 93 were added to different commercial nondairy drinks and viability and resistance to simulated gastric digestion (pH 2.5, 90 min, 37 °C) were monitored along storage (5 and 20 °C). For both strains, at least one nondairy drink was found to offer cell counts around 7 log orders until the end of the storage period. Changes in resistance to simulated gastric digestion were observed as well. Commercial probiotic cultures of *L. casei* can be added to commercial fruit juices after a carefull selection of the product that warrants cell viability. The resistance to simulated gastric digestion is an easy-to-apply *in vitro* tool that may contribute to product characterization and may help in the choice of the food matrix when no changes in cell viability are observed along storage. Sensorial evaluation is mandatory before marketing since the product type and storage conditions might influence the sensorial properties of the product due to the possibility of growth and lactic acid production by probiotic bacteria.

Keywords: fruit juice, gastric resistance, nondairy drink, probiotic, storage, viability

Practical Application: Many probiotic cultures are available for application in dairy products. However, care must be taken before applying them to different foods and the necessary control of viable cells must be carried out in order to diversify the market of probiotic products with the present available commercial strains.

Introduction

Probiotics have been defined as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO 2002). Fermented dairy products, such as fermented milks and fresh cheeses, have been the food vehicles with the biggest technological and commercial success for the incorporation of probiotic bacteria (Saxelin 2008; Figueroa-González and others 2011). However, with an increase in the vegetarianism as a dietary habit throughout the world and the increased allergy episodes against dairy proteins, there is also a demand for probiotics by nondairy consumers. Furthermore, lactose intolerance and the cholesterol content of dairy products can be considered perhaps two major drawbacks related to fermented dairy foods (Prado and others 2008.). In any case, dairy and nondairy consumers are still interested in consuming probiotics for their perceived beneficial health effects (Ranadheera and others 2010). There are already some relatively new nondairy probiotic beverages in the market. Grainfields Wholegrain Liquid® delivers active lactic acid bacteria (*Lactobacillus acidophilus* and *Lactobacillus delbrueckii*) and yeasts (*Saccharomyces boulardii* and *S. cerevisiae*) and it is made from grains, beans, and seeds (Superfoods 2006). Proviva®, a fruit drink, was the first probiotic food that does not contain milk, or milk constituents. Launched in Sweden, the product is composed by fermented oatmeal gruel and contains *Lactobacillus plantarum* 299v as probiotic adjunct (Molin 2001). Other nondairy probiotic products include Gefilus®, a fruit drink, developped by Valio Ltd. (Helsinki, Finland) that incorporates *L. rhamnosus* GG (Leporanta 2005a, 2005b); Bioprofit® with *L. rhamnosus* GG and *Propionibacterium freudenreichii* susbp. *shermanii* JS (Daniells 2006); Biola®, again a juice drink, manufactured by Tine BA in Oslo, Norway, also containing *L. rhamnosus* GG (Leporanta 2005b) or Rela®, a fruit juice with *L. reuteri* MM53 manufactured by Biogaia, Stockholm, Sweden (Prado and others 2008.). In some of these cases, a strain traditionally used in dairy products, such as *L. rhamnosus* GG, was succesfully included in fruit juices, where it was demonstrated to be stable during the refrigerated storage of the product. The incorporation of probiotic bacteria into a food matrix implies the necessity to maintain viable cells from production to consumption, where the cold chain plays a relevant role for the maintenance of viability (Ross and others 2005). Probiotic cell viability depends on various factors such as the type of product (Birollo and others 2000), the chemical ingredients used (Vinderola and others 2002a), and the possible interactions among strains (Vinderola and others 2002b). Cell viability is important for cell functionality (Ouwehand and Salminen 1998) and at the same time cell functionality is highly influenced by the food matrix components (Ranadheera and others 2010). However, when only cell viability is regarded as a quality control parameter for

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commercial probiotic products, changes in cell functionality might occur without being detected by routine plate counts. In previous works, we demonstrated that cell viability of probiotic bacteria can be maintained in commercial fermented milks (Vinderola and others 2011) or baby foods (Vinderola and others 2012) but the resistance to simulated gastric digestion, as indicator of functionality, might change along storage as a function of time and product variety. The aim of this work was to assess the cell viability and resistance to simulated gastric digestion, of commercial probiotic strains commonly used in dairy products, in industrial fruit juices available in Argentina. The impact on sensorial properties of their addition was also assessed.

Materials and Methods

Strains

A frozen culture of *Lactobacillus casei* LC-01 (Chr. Hansen, Hørsholm Denmark, www.chr-hansen.com) and a freeze-dried culture of *L. casei* BGP 93 (Sacco S.R.L., Cadorago, Italy, http://www.saccosrl.it) were used. Both strains were reported in the literature, or declared by the manufacturers, as probiotic ones (Homayouni and others 2008; Boza and others 2010) and are recommended for use in fermented dairy products, such as fermented milks and cheeses (www.chr-hansen.com, http://www.saccobrasil.com.br/pfermentado.html). Strains were stored and used according to the manufacturer's instructions.

Commercial nondairy drink samples

The following nondairy drinks were used: anana (pH 4.11), orange (pH 4.10), multifruits (pH 4.13), and diet orange (pH 4.28) soy milk Ades; peach (pH 3.67) and multifruits (pH 3.70) juice Baggio; orange (pH 3.63), apple (pH 3.34), and multifruits (pH 3.74) juice Cepita; orange (pH 3.47) and citrus (pH 3.48) juice BC La Campagnola; apple-flavored drink Gatorade (pH 3.11) and orange fruit juice Citric (pH 3.50). The products were purchased at local supermarkets, stored at 5 °C and used within the same week of acquisition.

Screening of inhibitory capacity of nondairy drinks towardprobiotic lactobacilli

The well-diffusion agar assay was used. Briefly, 20 mL of MRS agar (Biokar, Beauvais, France) melted and tempered at 45 °C were vigorously mixed with 200 μL of a cell suspension of the strains under study adjusted to ca. 1×10^9 CFU/mL in phosphate buffered saline (PBS) solution (pH 7.4) and poured into Petri dishes. Wells of 10 mm in diameter were made in the agar layer, and 180 μL of the nondairy drinks listed previously were placed into each well in triplicate. Plates were incubated (72 h, 37 °C, aerobic incubation) and the halo of inhibition was measured.

Cell viability and resistance to simulated gastric digestion in fruit juices

In a first assay, both lactobacilli strains were added to multifruits Ades juice to attain an initial level of ca. 7 log orders. Juices were kept at 5 °C for 4 wk. Selective cell counts of probiotic lactobacilli were performed weekly in LP-MRS agar (Vinderola and Reinheimer 2000). MRS-LP agar composition is: MRS agar containing 0.2% (w/v) lithium chloride (Sigma, St Louis, Mo., U.S.A.) and 0.3% (w/v) sodium propionate (Sigma). Plates were incubated at 37 °C for 48 h in aerobiosis. Cell morphology was confirmed by light microscopy examination (1000×). The resistance to simulated gastric digestion was also performed weekly according to Vinderola and others (2011). Briefly, a volume (20 mL) of juice was mixed (1 : 1) with a "salive-gastric" resembling-solution. Salive-gastric solution contained $CaCl_2$ (0.22 g/L), NaCl (16.2 g/L), KCl (2.2 g/L), $NaHCO_3$ (1.2 g/L), and 0.6% (w/v) porcine pepsine (Merck, Darmstadt, Germany). A 1 mL sample was removed for cell counts immediately after mixture and pH was then 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 on MRS-LP agar for cell viability assessment.

In a second assay, only *L. casei* LC-01 was added to 4 different brands and flavours of fruit juices: orange juice Ades, apple juice Baggio, apple juice BC La Campagnola, and peach juice Cepita. Samples were kept at 5 °C for 4 wk. Cell viability and resistance to simulated gastric digestion were performed weekly as described previously.

In a 3rd assay, only *L. casei* LC-01 was added to peach juice Cepita. Samples were kept at 5 °C and at 20 °C (considered as room temperature) for 4 wk. Cell viability and resistance to simulated gastric digestion were performed after 48 h of inoculation and weekly (from the moment of the inoculation) as described previously. In this assay, the sensorial properties of the products were assessed as described as follows.

Sensorial evaluation of peach juice added with *L. casei* LC-01

Sensory characteristics of juice and its evolution over time were evaluated according to the Difference from Control Test (Lawless

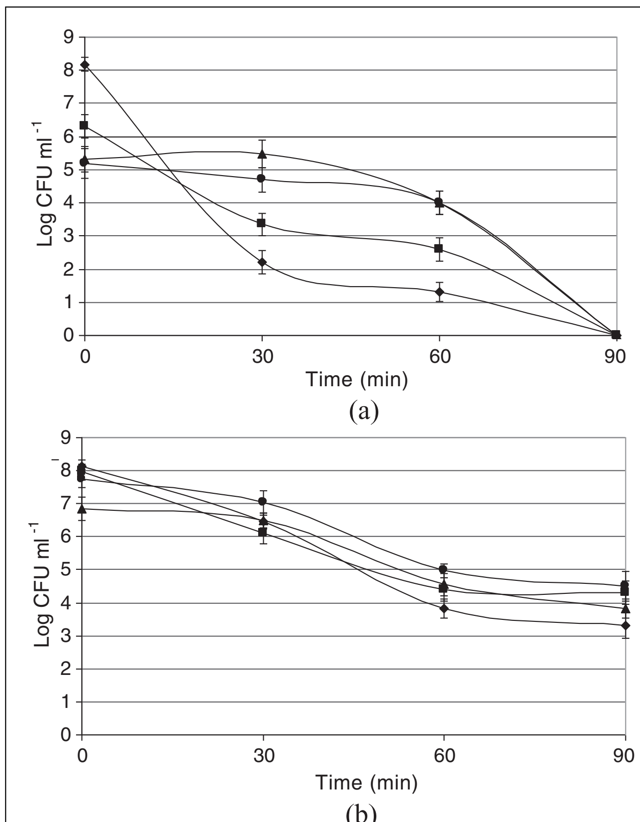

Figure 1–Cell viability of *L. casei* LC-01 (a) and *L. casei* BGP 93 (b) during the simulated gastric digestion (90 min) in multifruits Ades juice along 0 (♦), 7 (■), 21 (●), and 28 (▲) days of storage at 5 °C.

and Heymann 2010), considering odor, color, sweetness, acidity, and homogeneity. These attributes were selected by consensus from descriptors proposed for the analysis of orange and tangerine juices (Rega and others 2005 ; Carbonell and others 2007). Twenty presentations of each sample with the corresponding labeled control were assessed by a panel of 10 members, selected and trained according to ISO 2008. A numerical category scale of 9 points was used. Samples were served at room temperature, arranged in plastic cups containing 40 mL of product, simultaneously with a fresh juice sample labeled as control. Mineral water was supplied as palate cleanser between samples. Samples were assayed at 0, 6, 13, 20, and 27 d after inoculation and stored at 5 and 20 °C.

Statistical analysis

All experiments were conducted in duplicate by 2 independent groups of 2 operators each and cell counts were performed in duplicate. Commercial nondairy drinks were purchased once in order to avoid possible interference between different industrial batchs of the same product, a variable that cannot be controlled within the study. Data were analyzed using the one-way ANOVA procedure of SPSS software. The differences among means were detected by the Duncan's multiple range test. Data were considered significantly different when $P < 0.05$. The sensory data were submitted to analysis of variance and principal component analysis (PCA) of SAS v.9.3 software.

Results and Discussion

One way to address the growing demand for probiotics is the diversification of the food matrices used to deliver these beneficial bacteria. The increased varieties of probiotic foods, beyond fermented dairy products, will certainly satisfy consumers with specific demands or needs such as strict vegetarians, lactose intolerants, milk-protein allergics, or those consumers with a negative perception of dairy products, specially cheeses, due to their salt and cholesterol content. There is a genuine interest in the development of fruit-juice-based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods (Rivera-Espinoza and Gallardo-Navarro 2010; do Espirito Santo and others 2011). However, the commercial and functional success of new food matrices carrying probiotic bacteria will depend on factors such as the stability of probiotic bacteria in the product and on the frequency with which the food is consumed: no beneficial effects should be expected if probiotic bacteria are added to foods that are consumed rarely or on an irregular basis (once a week, twice or 3 times a month, for example).

The growth inhibitory capacity of 13 nondairy drinks was assessed against 2 probiotic commercial strains of *L. casei*, by means of the well-diffusion agar assay. Only citric orange juice affected the growth of both strains under study as revealed by the presence of an inhibition halo (6 to 7 mm for both strains, measured from the edge of the well).

Cell counts of *L. casei* LC-01 gradually diminished along storage from ca. 8 log orders to 5 log orders (see values of counts at time 0, Figure 1a). However, the profile of resistance to gastric digestion gradually increased, although no viable cells were observed by the end of the simulated gastric digestion. When studying the gastric resistance of probiotic strains, some puzzling results have been reported. For example, strains with a well-documented ability to perform beneficially on the human gut, scored poorly in *in vitro* assays of gastric acid resistance. The discrepancy between

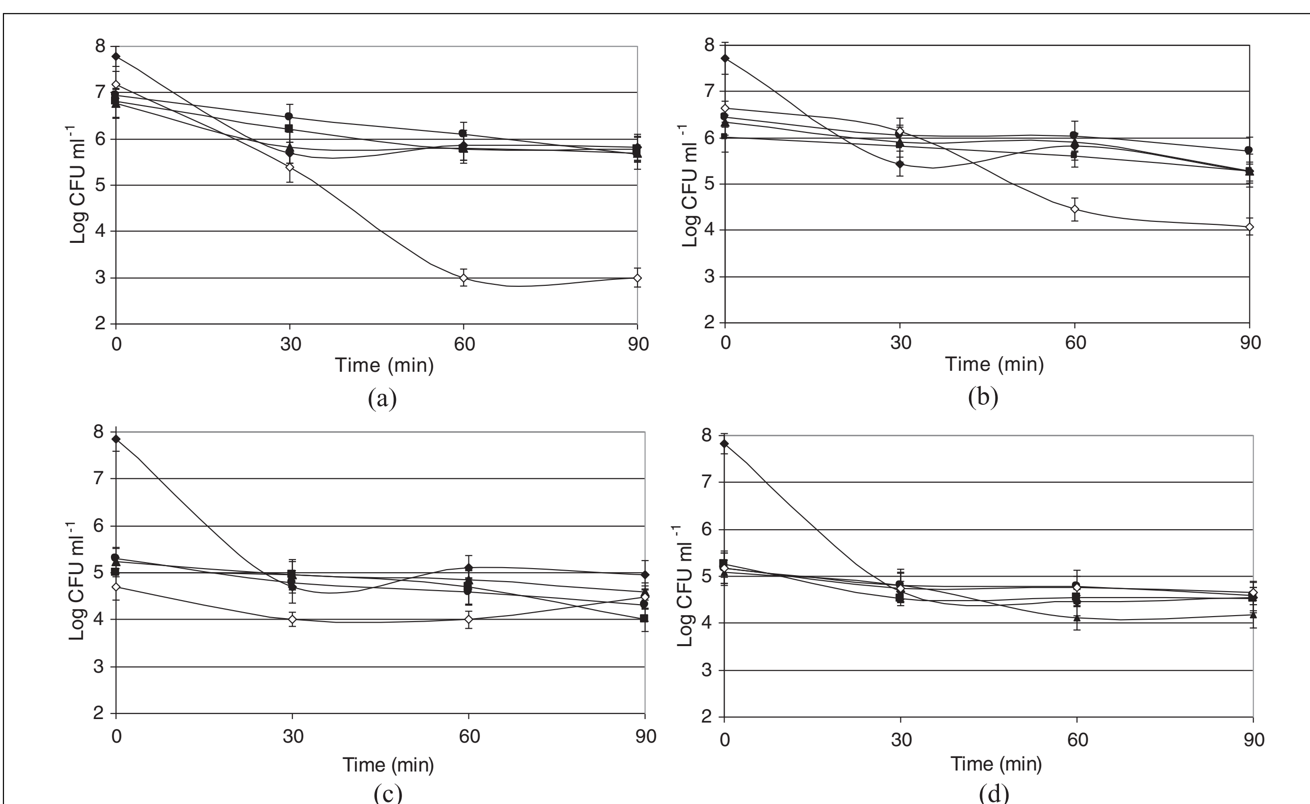

Figure 2–Cell viability of *L. casei* LC-01 during the simulated gastric digestion (90 min) in orange juice Ades (a), apple juice Baggio (b), apple juice BC La Campagnola (c), and peach juice Cepita (d) along 0 (♦), 7 (■), 14 (▲), 21 (●), and 28 (◇) days of storage at 5 °C.

in vitro and *in vivo* might suggest the need still for further refined tests to estimate *in vitro* the *in vivo* resistance to gastric digestion (Morelli 2007). However, *in vitro* tests are still useful to study the impact of some technological factors, such as storage or food matrix, on the gastric resistance of probiotic bacteria, as observed by us in previous studies (Vinderola and others 2011; Vinderola and others 2012). Cell counts of *L. casei* BGP 93 along storage in multifruits Ades juice maintained close to the inoculated value (ca. 8 log orders) during the first 3 wk of storage. One log cycle of cell decay was observed by the 4th wk (Figure 1b). During the simulated gastric digestion, a cell decay of ca. 4 log cycles was observed at time 0 and a cell decay of 3 log cycles was observed from weeks 1 to 4 of storage. Resistance of *L. casei* BGP 93 to simulated gastric digestion significantly increased in 1 log order by the 1st wk of storage and maintained until its end. Pre-exposure to sublethal levels of a stress factor has been shown to allow cells to adapt to subsequent exposure to higher levels of the same stress factor or to different stresses, a phenomena called cross adaptation (Bunning and others 1990; O'Driscoll and others 1996). In this context, the higher resistance of lactobacilli to simulated gastric digestion achieved along storage might be due to the exposure to the acidic conditions of juices during refrigeration. In line with these findings, in previous studies, we observed an enhanced resistance to bile salts in nonintestinal lactobacilli due to pre-exposure to gradually increased levels of bile (Burns and others 2008) and an enhanced resistance to simulated gastric digestion in probiotics in commercial fermented milks along storage (Vinderola and others 2011), in bifidobacteria grown at low pH values (Vinderola and others 2012) and in spray-dried lactobacilli due to preliminar heat-treatment and spray-drying (Páez and others 2012).

Since *L. casei* BGP 93 resulted more stable than *L. casei* LC-01 in this food matrix, further studies were conducted with the latter in order to find a suitable nondairy vehicle for this strain. Cell viability and resistance to simulated gastric digestion were studied for *L. casei* LC-01 in orange juice Ades, apple juice Baggio, apple juice BC La Campagnola, and peach juice Cepita along storage (Figure 2). Loss of cell viability of ca. 1 to 1.5 log orders after 1 wk of storage was observed in orange juice Ades (Figure 2a) and apple juice Baggio (Figure 2b), whereas a cell decay of ca. 3 log cycles was observed in apple juice BC La Campagnola (Figure 2c) and peach juice Cepita (Figure 2d). When considering the resistance to simulated gastric digestion, a more erractic behavior was observed depending on the product studied. Cells that remained alive after 1 wk of storage in apple juice BC La Campagnola and peach juice Cepita were resistant to simulated gastric digestion and the profile was similar along storage. In the products orange juice Ades and apple juice Baggio, cell decays due to simulated gastric digestion (1 to 2 log cycles) were observed along storage, except by day 28 were cell decays were higher (4 to 5 log cycles). The reduction of gastric resistance in lactobacilli along storage has been reported by Wang and others (2009) for *L. casei* depending on the food matrix considered. The authors reported that cells became more sensitive to simulated gastric acidity with storage in bovine milk but not in soy milk. Saarela and others (2006) also reported a progressive decrease of resistance to simulated gastric acidity of bifidobacteria maintained in fruit juice at 4 and at 20 °C for 6 wk. It is interesting to note that in the referenced studies and in this work, there was no loss of probiotic cell viability during storage, but a decrease of cell resistance to gastric acidity, showing and uncoupling between cell viability and cell functionality (regarded as the resistance to simulated gastric digestion). Considering these results, *L. casei* LC-01, a strain considered to be potentially probiotic (Liu and others 2006; Homayouni and others 2008), resulted to be instable in certain fruit juices in this study. Fruit juice is a food category more heterogeneous regarding the physicochemical properties than fermented milks. Then, it is not surprising that the survival of probiotic strains in these products be very variable and product-dependent. According to Sheehan and others (2007), when adding *Lactobacillus* and *Bifidobacterium* to orange, pineapple, and cranberry juice, extensive differences regarding their acid resistance were observed. Saarela and others (2006) reported that sucrose-freeze-dried cells of *Bifidobacterium animalis* subsp. *lactis* survived better in juice than skim milk-freeze-dried cells. Yoon and others (2004) reported that *L. acidophilus*, *L. plantarum*, *L. casei,* and *L. delbrueckii* survived in fermented tomato juice with low pH for 4 wk at 4 °C. But later on, the same group reported that *L. casei* lost cell viability completely after only 2 wk in cabbage juice (Yoon and others 2006). Kyung and others (2005) reported that *L. acidophilus* was considerable less stable in fermented beet juice than *L. plantarum* or *L. casei*. Charernjiratrakul and others (2007) reported that 5 strains of *L. plantarum* lost about 2 log cycles of cell viability after 15 d of cold storage in carrot juice. In general, according to the probiotic studies reported, the growth and the viability of cells in fruits and vegetables depend on the strains used, final acidity, and the concentration of the organic acids of the product (Rivera-Espinoza and Gallardo-Navarro 2010). Anyway, technological changes and product development can be carried out in order to make a strain suitable (adapted) for other food matrices. For example, it was informed that *L. paracasei* 431, a strain with scientific documentation on its probictic characteristics

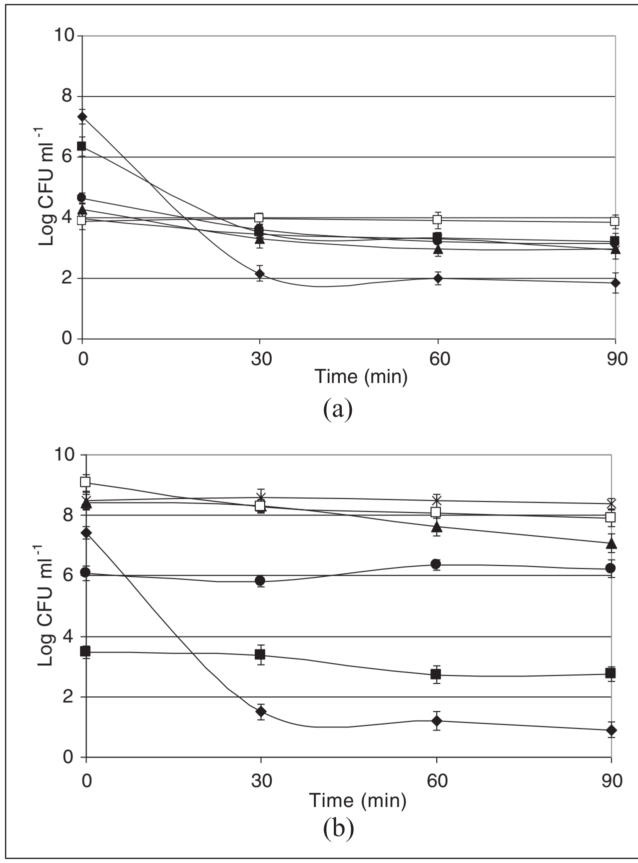

Figure 3–Cell viability of *casei* LC-01 during the simulated gastric digestion (90 min) in peach juice Cepita at time 0 (♦), 48 h (■), 7 (●), 14 (▲), 21 (×), and 28 (□) days of storage at 5 °C (a) or at 20 °C (b).

Table 1–Coefficients of the first 2 principal components F1 and F2 in the original matrix for sensory variables.

	Odor	Color	Sweetness	Acidity
F1	0.532	−0.452	0.517	−0.496
F2	0.077	0.828	0.126	−0.541

demonstrated in human trials (Lee and Salminen 2009) and long history of use in yoghurt and dietary supplement products, was recently developed to survive in low pH environments such as chilled juice and juice drinks (http://www.chr-hansen.com/news-media/singlenews/add-the-good-life-to-juice.html). Some strategies recently proposed to increase the survival of sensitive probiotic strains in fruit juices include the use of oat fiber and low pH selection combined or not with UV mutagenesis (Saarela and others 2011a, 2011b b).

Finally, in our study, *L. casei* LC-01 was added to peach juice Cepita and kept at 5 and 20 °C (room temperature). Additionally, cell counts were performed after 48 h of its addition to the fruit juice. As in the previous case, a cell decay of ca. 3 log cycles in this product was observed after 1 wk of storage at 5 °C (time 0 of simulated gastric digestion, Figure 3a). Again, the cells that remained viable after 1 wk were resistant to simulated gastric digestion. An enhanced resistance to this stress factor (1 to 2 log cycles) was observed after 1 wk of storage when compared to the profile observed at time 0. In samples stored at 20 °C (Figure 3b). a 2 log cycle decay was observed after 48 h of storage, but, from that point onward, an increase in cell counts was observed. By day 14 of storage, cell counts ranged from 8 to 9 log cycles. Consequently, the pH of the product dropped from 3.94 to 3.27. When considering the resistance to simulated gastric digestion, 5 log orders of cell decay were observed in cells at time 0. However, from hour 48 of storage and onward, an enhanced resistance to simulated gastric digestion was observed since no cell death was observed when cells were exposed to pH 2.50 in the presence of porcine pepsin.

Considering the sensorial evaluation of juices, modifications in their sensory characteristics were observed due to the presence of the probiotic cultures. From the beginning, the juice stored at 5 °C showed higher acidity and less sweetness than the control. Over the assay, these features were accentuated. The smell was fading and changing its qualitative characteristics, passing fruit (peach, banana), preserved fruit, ripe, cooked, stewed, and ending in the appearance of off-flavors, agreeing with Krasaekoopt and Kamolnate (2010), in juices containing alginate-microencapsulated probiotics. These changes would impact adversely on the degree of satisfaction of consumers, who acquire this product for flavor and nutritional characteristics (Luckwow and Delahunty 2004a ,2004bb).

Samples stored at 20 °C showed a deterioration greater than those stored at 5 °C, especially the odor (or smell), from day 13, and in taste from day 20. The juice also darkened in the course of time and stabilized from day 20, remaining unchanged until the end of the assay.

PCA was carried out on the basis of the data matrix after standardization. The matrix contained the information of the sensory variables: intensity of odor, color, sweetness, and acidity. The eigenvalues for the first 2 principal components (*F*) were $\lambda_1 = 3.47$ and $\lambda_2 = 0.42$, which explained jointly the 97% of the total variation of the data, an acceptable criterion to represent 2 new latent variables of the original information. The coefficients of the first 2 principal components are shown in Table 1.

In relation to the PCA for the sensory analysis (Figure 4), it can be seen a displacement of the samples to the left in direction to the first component (F1), as time passes from day 0 to day 27 in relation to the decrease in sweetness and smell. In the direction of the second component (F2), the samples moved mainly according to the color increase. However, the first component retained 87% of the variation, indicating the major changes. The load factor of acidity was also primarily related to the 1st component. Sensory changes indicated that the shelf life of inoculated juice would not exceed 1 wk under the conditions studied. This is consistent with other studies reporting that inoculated juices were completely unacceptable to ordinary consumers, but were tolerated by those who rarely or never consumed (Krasaekoopt and others 2008).

In Argentina, the issue of probiotics in foods has been incorporated in the Argentinian food code in December 2011 (www.anmat.gov.ar/alimentos/normativas_alimentos_caa.asp).

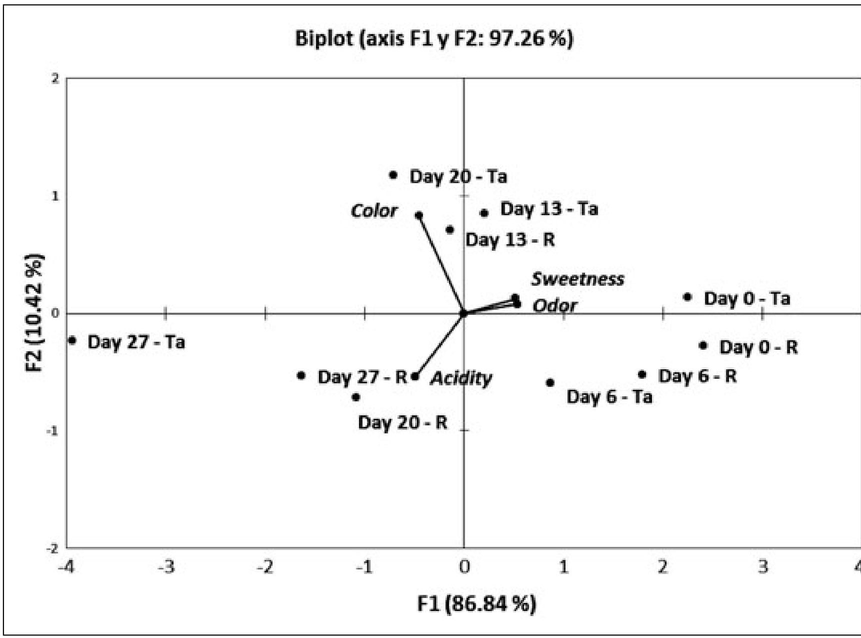

Figure 4–Bidimensional projection of principal component analysis of peach juice carrying *L. casei* LC-01 stored for 28 d at 20 °C (Ta) and 5 °C (R).

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However, there is no official regulation (nor control) about the minimal content of probiotic bacteria in foods yet. It is likely that the required amount could be different between different probiotic strains in different foods since functional effects are strain- and dose-dependent (Vinderola and others 2005; Minelli and Benini 2008; Fang and others 2009). The food matrix might also influence the functional effects of probiotics (Ranadheera and others 2010). There is an international trend or consensus to admit that any food should contain at least 10^7 to 10^8 CFU/mL in order to exert a health benefit (Champagne and Gardner 2005). Additionally, the majority of human studies were carried out with products containing probiotic bacteria within this range (Montrose and Floch 2005). In this context, for both strains considered in this study, at least one nondairy drink was found to offer cell counts close to 7 log orders until the end of the refrigerated storage period considered. In the other nondairy products studied, the instability of cell counts along storage makes it necessary to consider each food matrix in particular before adding probiotics and to carry out the necessary controls, avoiding the extrapolation of promising results from one strain to another or from one food product to another. Even when fermented milks have been pointed out as the food vehicles with the biggest success for the incoporation of probiotic bacteria due to the stability that viable cells can achieve (Saxelin 2008; Figueroa-González and others 2011), reports of unsatisfactory counts of probiotic bacteria during cold storage of the products were reported as well in Australia (Micanel and others 1995; Shah and others 1995), Germany (Schillinger 1999), Argentina (Vinderola and others 2000), Italy (Fasoli and others 2003), Spain (Gueimonde and others 2004), and France (Coeuret and others 2004). These background information and our results continue to support the fact that probiotic stability in a given product is a strain- and specific-product feature.

Conclusion

Commercial probiotic cultures of *L. casei* can be added to commercial fruit juices after a carefull selection of the specific product that warrants cell viability. The resistance to simulated gastric digestion is an easy-to-apply *in vitro* tool that may contribute to product characterization and may help in the choice of the food matrix when no changes in cell viability are observed along storage. The instability of the cultures observed in certain products justify the efforts needed to find the right food matrix and highlights the impossility to extrapolate results between strains or similar products. Sensorial evaluation is mandatory before marketing since the product type and storage conditions might influence the sensorial properties of the food.

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