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GASTROINTESTINAL SURVIVAL OF BACTERIA IN COMMERCIAL PROBIOTIC PRODUCTS

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ABSTRACT: ***This work compared bacterial gastrointestinal (GI) resistance of commercial probiotic products (capsules, fermented milk and powder). To simulate GI transit, the probiotic products were subjected to gastric fluid for 120 min then to intestinal fluid for 180 min. Gastric and intestinal fluids were prepared according to United States Pharmacopeia protocols. Bacterial enumeration was compared before and after the GI transit to evaluate the protective effect of the vehicle or the food matrix. Bacteria of the four probiotic capsules covered with an enteric coating had a higher survival rate (<1 log_{10} CFU reduction) than uncoated. Eight encapsulated but non enteric coated probiotic products showed limited GI resistance (between 1 and 5 log_{10} CFU reduction) while five products showed no GI survival. For probiotic fermented milk, two products demonstrated excellent or good protective property (<1 log_{10} CFU reduction) while the other four showed no resistance. Only one of six powdered probiotic strains had excellent GI survival. This study demonstrated that GI survival varies from one probiotic product to another. It reiterates the importance of manufacturing probiotic strains using the appropriate vehicle for the bacteria to reach its site of action and produce the expected beneficial effects.***

KEY WORDS: Acid Tolerance, Bile Salts, Gastrointestinal, Probiotic

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INTRODUCTION

Probiotics are defined as «live microorganisms which, when administered in adequate amounts, confer a health benefit on the host» (Araya et al. 2002). A good probiotic strain should preferably be of human origin, possess a generally recognized as safe (GRAS) status, the capacity to survive through the gastrointestinal (GI) tract and colonize the gut (Ronka et al. 2003). A wide range of probiotics ready for consumption are currently available on the market. However, the efficacy of commercially available probiotic products differs a lot, since their properties and characteristics are different from a probiotic strain to another. In most cases, marketing has preceded scientific control (De Angelis et al. 2007). In fact, the GI survival of several strains of probiotics has not been supported by scientific evidence. In order for the bacteria to exert their beneficial effects on the host, they must be able to survive and reach the GI tract in sufficient numbers, at least 10^6-10^7 CFU/g (Bosnea et al. 2009). The ability of a probiotic to survive through the GI system depends mainly on their acid and bile tolerance. During GI passage, the strains are required to tolerate the presence of pepsin and the low pH of the stomach, the presence of enzymes in the duodenum and the antimicrobial activity of bile salts (Masco et al. 2007). Therefore, it is indispensable to demonstrate their survival by *in vitro* experiments that simulate the human GI tract conditions before conducting expensive *in vivo* tests.

The most studied probiotic are the lactic acid bacteria (LAB), especially *Lactobacillus* and *Bifidobacterium* (Verdenelli et al. 2009). They are also the most commonly found in probiotic products for human consumption (Gueimonde et al. 2004; Masco et al. 2007). Lactobacilli are non-pathogenic microorganisms in human and animal intestine. Studies have shown that lactobacilli possessed inhibitory effect towards enteropathogens and produce several antimicrobial compounds (Jacobsen et al. 1999; Millette et al. 2007). *Bifidobacterium* strains have also various health benefits, from inhibition of enteric pathogens to amelioration of lactose digestion, immune system modulation, and reductions of symptoms related to allergy and hepatic encephalopathy (Talwalkar and Kailasapathy 2004).

The biggest issue regarding many *in vitro* studies is that these experiments do not evaluate the GI survival rate of probiotic strains in commercial products. In 2008, Sumeri et al. reported that the same probiotics in different food matrix behaved differently. This, together with variations in bile excretion between individuals and with the food, could clarify the contradictory results obtained between *in vitro* and *in vivo* experiments.

A recent study demonstrated that *Lactobacillus casei* Shirota, *L. casei* Immunitas and *L. acidophilus* subsp. *johnsonii* were able to survive *in vitro* gastric and gastric plus duodenal digestion by using a dynamic gastric model (DGM) of digestion followed by incubation under duodenal conditions, with milk and/or water as vehicle. *L. acidophilus johnsonii* was found to be the best probiotic strain because of its highest survival in both tested foods (milk and water) (Lo Curto et al. 2011). A dynamic model with two reactors simulating gastric and duodenal conditions was designed by Mainville in 2005 (Mainville et al. 2005). A food matrix was included in the design to better represent the pH levels found *in vivo* before, during and after meal consumption. Two strains (*Bifidobacterium animalis* ATCC 25527 and *Lactobacillus johnsonii* La-1 NCC 533) exhibited good survival through the GI tract with and without the food matrix. Another simple and non expensive way to assess the GI survival of bacteria is to use static simulated gastric and intestinal fluids. In fact, another recent study demonstrated that bile-adapted *Bifidobacterium* strains were able to better survive *in vitro* in human gastric and duodenal fluids than the wild strain (de los Reyes-Gavilan et al. 2011). Moreover, Millette *et al*. (2008) used this model to demonstrate the GI survival of various probiotics.

Therefore, the aim of the present study was to establish the GI resistance *in vitro* of the bacteria contained in 29 commercially available probiotics. To our knowledge, this is the first study verifying the GI survival of probiotic bacterial strains in finished commercial product as available in the market. This is of importance because viability is part of the WHO/FAO probiotic definition. To mimic the GI conditions, simulated gastric and intestinal fluids have been used.

MATERIAL AND METHODS

Commercial probiotic products

Twenty-nine commercially available probiotic products were purchased from natural health food stores, supermarkets or drugstores in USA and Canada. All tests were performed using the commercial product (fermented milk, powder, capsules and yogurts) as purchased. The probiotic products were stored as recommended on their label (room temperature or refrigerated) until utilization. Strains labelled on the probiotics are presented in the Table I (capsules) or in Figures 2 (fermented milks or probiotic-enriched yogurts) and 3 (powders).

TABLE 1. Ability of capsules to remain intact after 2 h in simulated gastric solution, pH 1.5.

Probiotic Capsule	Number of capsules resistant to gastric acidity after 2 h	Strains
1	6/6	*L. acidophilus* CL1285, *L. casei* LBC80R
2	6/6	*B. bifidum, B. breve, B. longum, L. acidophilus, L. rhamnosus, L. casei, L. plantarum, Lc. lactis, L. bulgaricus, L. salivarius*
3	6/6	*L. bifidus, L. acidophilus, L. helveticus* 8781, *L. plantarum, L. casei, B. longum, B. infantis, B. breve, S. thermophilus, L. bulgaricus*
4	6/6	*L. rhamnosus* R0011, *L. casei* R0215, *L. plantarum* R1012, *L. acidophilus* R0052, *B. longum* BB536, *B. breve* R0070, *P. acidilactici* R1001, *Lc. lactis* R1058
5	0/6	*B. bifidum* HA-132, *B. longum* HA-135, *B. breve* HA-129, *L. acidophilus* HA-122, *L. casei* HA-108, *L. rhamnosus* HA-111, *L. rhamnosus* HA-114, *L. plantarum* HA-119, *Lc. lactis* HA-136, *S. thermophilus* HA-110
6	0/6	*L. acidophilus* R0052, *L. rhamnosus* R0011, *S. thermophilus* R0083, *Lc. lactis* R1058, *B. breve* RR0070, *B. longum* R0175, *P. acidilactici* R1001, *L. delbrueckii* R9001
7	0/6	*Saccharomyces boulardii, L. plantarum, Bacillus subtilis, L. paracasei, L. brevis, L. acidophilus, L. casei, L. rhamnosus, L. salivarius, B. longum, B. bifidum, B. breve, B. lactis*
8	0/6	*L. acidophilus, L. acidophilus, B. bifidum, B. lactis*
9	0/6	*L. acidophilus, L. casei, L. rhamnosus, Enterococcus faecium*
10	0/6	*L. rhamnosus, L. casei, L. acidophilus, B. longum, B. bifidum*
11	0/6	*L. acidophilus, L. rhamnosus, S. thermophilus, L. plantarum, B. bifidum, L. bulgaricus, B. longum, L. Salivarius*
12	0/6	*L. casei, L. rhamnosus, B. breve, B. longum, L. acidophilus, L. plantarum, L. rhamnosus, B. bifidum, Lc. Lactis, L. bulgaricus, L. helveticus, L. salivarius*
13	0/6	*L. rhamnosus* GG
14	0/6	*L. acidophilus* KS-13, *B. bifidum* G9-1, *B. longum* MM-2
15	0/6	*L. acidophilus, L. plantarum, L. rhamnosus, L. casei, L. paracasei, L. salivarius, B. bifidum, B. longum*
16	0/6	*L. acidophilus, L. rhamnosus, S. thermophilus, Lc. lactis, B. bifidum, B. longum, L. bulgaricus*
17	0/6	*L. acidophilus* LA-5, *B. lactis* BB12, *S. thermophilus* STY-31, *L. delbrueckii* LBY-27

Preparation of simulated gastric and intestinal fluids

To test the GI survival of encapsulated probiotic bacteria, a simulated gastric solution (SGF #1*)* at pH 1.5 was prepared (Anonymous 1995). This solution was prepared by dissolving 2.0 g of NaCl (Laboratoire MAT, Quebec, QC, Canada) and 3.2 g of porcine mucosa pepsin (1100 U/mg of protein; P-7000; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) in 900 mL of water. The pH was then adjusted by HCl (1 N; Fisher Scientific Company, ON, Canada) to obtain a final pH of 1.5. The solution was completed with water for a final volume of 1000 mL. The second simulated gastric solution (SGF #2) was needed for the treatment of probiotic fermented milk or yogurts and powders because all bacteria were killed by SGF at pH 1.5 as demonstrated in preliminary experiments. The formulation was similar as SGF #1, but the final pH was adjusted at 2.0 with HCl.

Finally, a simulated intestinal solution (SIF) was prepared by dissolving 6.8 g of KH_2PO_4 (Laboratoire MAT) in 250 mL of water. Then, 77 mL of NaOH (0.2 N) and 500 mL of water,1.25 g of pancreatin (activity equivalent to 8 times the specifications of USP; P-7545; Sigma-Aldrich) and 3 g of bile salts (Oxgall; P-8381; Sigma-Aldrich) were added to the solution. Eventually, the pH was adjusted to 6.8 ± 0.1 with NaOH (0.2 N) or HCl (0.2 N). The SIF was completed with water to obtain 1000 mL.

All the solutions were tested for sterility on MRS (EMD Chemicals inc, Mississauga, ON, Canada) and Plate Count agar (BD Biosciences, Mississauga, ON, Canada) and the plates were incubated for 72h at 37°C under anaerobic atmosphere.

Treatment of probiotic capsules in SGF

The SGF was incubated at 37°C for 60 min before the experiment to simulate the body temperature. A probiotic capsule was added to 25 mL of SGF #1 and then the solution was incubated at 37°C with stirring (200 rpm) using an incubator-shaker (Environmental Shaker G24, New Brunswick Scientific Co. Inc.; Edison, NJ, USA) to simulate the bowel movements. After 120 minutes, the capsule was removed and added to the SIF. If the capsule was dissolved, 1 mL of the gastric fluid was transferred to the SIF.

Treatment of probiotic fermented milk, powder or yogurts in SGF

The SGF #2 was incubated at 37°C for 60 min before the experiment to simulate the body temperature. One g of probiotic yogurt, fermented milk or powder was added to 24 mL of SGF #2, and the solution was incubated at 37°C under stirring (200 rpm) using an incubator-shaker (Environmental Shaker G24) to reproduce the bowel movements. After 120 minutes, 1 mL of the SGF#2 was transferred to the SIF.

Treatment of the probiotic products in SIF

The SIF was incubated at 37°C for 60 min before the experiment to simulate the body temperature. Following the gastric treatment, the 1 mL of SGF or the capsule taken previously was transferred in 24 mL of SIF. The intestinal suspensions were incubated at 37°C under stirring (200 rpm) for 180 minutes and 1 mL of each suspension was withdrawn and the evaluation of bacteria survival was performed as described below.

Assessment of bacterial survival

To determine the initial count of bacteria contained in the capsules, each non treated capsule was opened and rehydrated in 9 ml of MRS for 30 minutes at 37°C to allow optimal suspension of bacteria mixed with the excipients. Then, a series of tenfold dilution was performed in sterile peptone water (0.1% w/v) and appropriate dilutions were pour plated into MRS agar and incubated 72 h at 37°C under anaerobic conditions. The incubation time of 30 min did not allowed cell division of bacteria. Therefore, there was no risk of false results.

When powder, fermented milk or yogurts were evaluated, 11 g of product was added to 99 mL of sterile peptone water (0.1% wt/vol) in a sterile bag and homogenized using a Lab-blender 400 stomacher (Laboratory Equipment, London, UK) for 1 min. The suspension was diluted, plated and incubated as described above. The colonies were then enumerated using a Dark field Quebec Colony Counter.

After GI treatment, 1 mL of intestinal fluid was withdrawn then diluted in sterile peptone water, plated, incubated and enumerated as described above.

Statistical analysis

For each probiotic product, total bacterial concentration was evaluated from three independent samples before GI transit while six samples were subjected to GI fluids and analyzed for bacterial concentration per capsule or gram. Values are given as means ± standard deviation. Data were analyzed with the SPSS software (version 19; IBM-SPSS, Chicago, Ill, USA). Student's *t*-test for two paired samples was used to compare the mean of bacterial concentration of each probiotic product before GI treatment to the mean after the treatment. Differences between means were considered significant at $P \leq 0.05$.

RESULTS

Survival of probiotic capsules under GI conditions

To assess the resistance of probiotic capsules to gastric acidity, the products were added to SGF (pH 1.5) for 2 h. To determine the survival level of bacteria under GI conditions, the assessment of their survival was performed at the initial time (T = 0) and at the end of the intestinal time treatment. The difference between the two values was evaluated. Results showed that only probiotic capsules #1 to 4 were able to resist gastric acidity (< 1 $\log_{10}$ CFU reduction). Eight encapsulated but non enteric coated probiotic products showed limited GI resistance (between 1 and 5 $\log_{10}$ CFU reduction) while the last five products showed no GI survival. The other capsules were all dissolved under gastric condition (Table I and Figure 1).

Survival of fermented milk or probiotic-enriched yogurt under GI conditions

As for the fermented milk, only one out of the eight products evaluated (#18) demonstrated an excellent survival rate with an initial bacteria count of 8.98 log CFU/g and a final count of 9.00 log CFU/g (Figure 2). Another probiotic product showed a good survival (#19) with an initial count of 8.77 log CFU/g and a final count of 8.11 log CFU/g. The products #20-22 had a moderate GI survival with a respective initial value of 7.58, 7.23 and 6.47 log CFU/g and final counts of 5.47, 5.37 and 5.46 log CFU/g. The last fermented milk (#23) had a bad survival rate because its initial and final bacteria count was from 4.07 to 3.8 log CFU/g.

FIGURE 1. Survival of encapsulated probiotic bacteria after 2 h in simulated gastric fluid (pH 1.5) and 3h in simulated intestinal fluid (pH 6.8). An asterisk means significant difference between bacterial before and after GI treatment (P ≤ 0.05). Please see Table 1 legend for the type of bacteria in each capsule numbered 1 to 17.

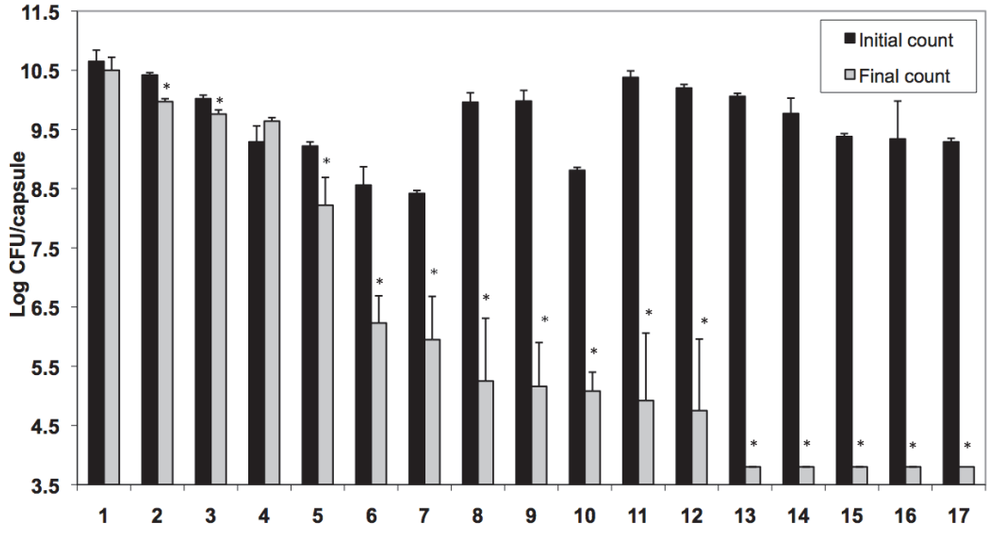


FIGURE 2. Survival of bacteria in fermented milk or probiotic-enriched yogurt after 2 h in simulated gastric fluid (pH 2.0) and 3h in simulated intestinal fluid (pH 6.8). 18: *L. acidophilus* CL1285 and *L. casei* LBC80R; **19:** *L. casei* DN-114 001; **20:** *B. lactis* DN-173 010; **21:** *L. acidophilus* NCFM and *B. lactis* HN 019; **22:** *B. lactis* and *L. acidophilus*; **23:** *B. lactis, Streptococcus thermophilus, L. bulgaricus, L. casei* and *L. acidophilus*. An asterisk means significant difference between bacterial before and after GI treatment (P ≤ 0.05).

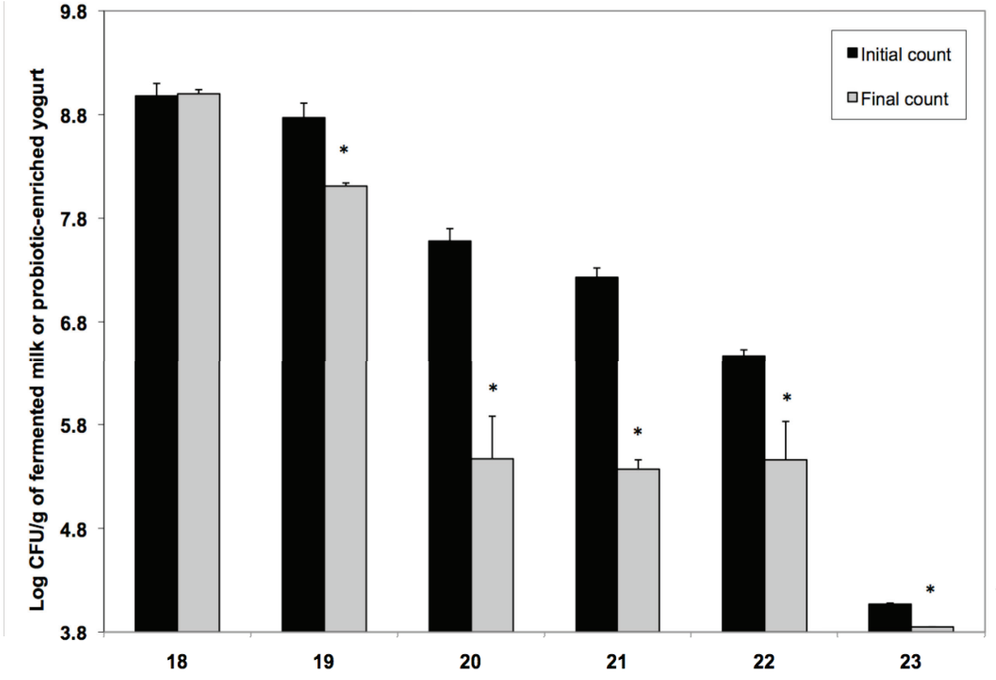
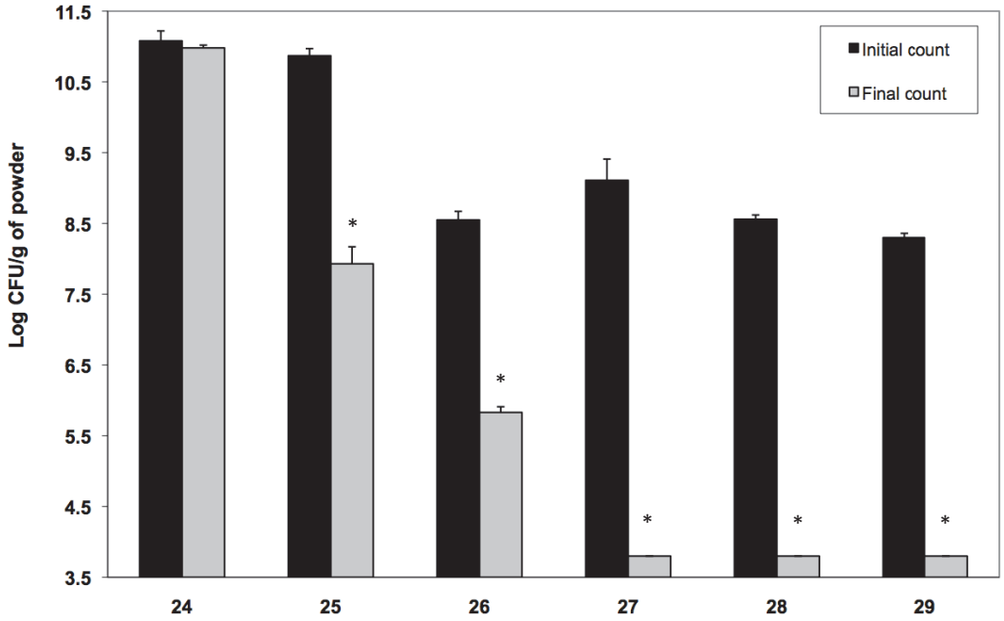


FIGURE 3. Survival of probiotic powder after 2 h in simulated gastric fluid (pH 2.0) and 3h in simulated intestinal fluid (pH 6.8). **24:** *L. casei, L. plantarum, L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. longum, B. breve*, *B. infantis* and *Streptococcus salivarius* subsp. *thermophilus*; **25:** *L. acidophilus*; **26:** *L. acidophilus* and *L. bifidus*; **27:** *B. longum* BB536; **28:** *L. acidophilus* LAC361 and *B. longum* BB536; **29:** *L. plantarum* and *B. lactis*. An asterisk means significant difference between bacterial before and after GI treatment ($P \leq 0.05$).



Survival of probiotic powder under GI conditions

Six probiotic powders were evaluated for their GI survival (Figure 3). Results showed that the product #24 was the only one showing an excellent survival rate with an initial count of 11.08 log CFU/g and a final count of 10.98 log CFU/g. The samples #25 and #26 had a moderate survival rate showing an initial count of 10.87 and 8.55 log CFU/g and a final counts of 7.93 and 5.83 log CFU/g respectively. The last three probiotic powders (#**27-29**) demonstrated a bad survival rate by having a respective initial value of 9.11, 8.56 and 8.3 log CFU/g and a final count of under the limit of detection (3.8 log CFU/g) for each of them.

DISCUSSION

Although many scientists agree on the importance of the probiotics bacteria survival *in vivo*, many products available on the market don't meet the requirements. This study demonstrated that not all probiotic products were able to survive GI conditions *in vitro*, and showed that among the probiotic capsules evaluated, only those that were enteric coated were able to resist to the degradation caused by stomach conditions. The results demonstrate the importance of protecting the bacteria by adding an enteric coating to the capsules. These data also support those found by Priya *et al.* (2011). These authors showed that the GI survival of *L. acidophilus* increased when the probiotic was encapsulated. In fact, the uncoated bacteria were almost completely destroyed under GI conditions. Moreover, the encapsulated bacteria are freeze-dried to increase the bacterial concentration and the stability of the probiotic products. This study confirm also that enteric coating protect the bacteria during their passage through the GI tract because its ingredients resist dissolution under acidic conditions, but are soluble under the alkaline conditions of the intestine (Long and Chen 2009). However, several studies have reported that the conditions under which samples are freeze-dried (e.g. phase of growth, suspending fluid, cell concentration, drying and freeze-drying technique) could strongly affect the bacterial viability (Berny and Hennebert 1991; Lodato et al. 1999; Bolla et al. 2011). Therefore, it is important to assess the survival of probiotic strains by evaluating the final product.

For the probiotic powders, only one product had an excellent survival rate (#24). Compared to the other samples, that product contained a higher level of bacteria, with 450 billion live bacteria per package. It could be hypothesized that the large amount of bacteria in the product may have a protective effect, which would explain the great survival of the probiotic strains.

One probiotic milk (#18) stood out from the others because of its excellent rate of GI survival. This product was a fermented milk unlike other products that were probiotic-enriched yogurt. The advantage of fermented substances is that the exogenous bacteria reach the large intestine in an intact and viable form, which allows them to exert their effect immediately upon consumption. Therefore, this protective and nourishing environment could ensure optimal bacterial activity (Gibson and Roberfroid 1995). In addition, some studies have shown that probiotic strains survived better when stored in milk (Lo Curto et al. 2011; Tompkins et al. 2011).

This result could be related by the buffering effect of milk which could protect the strains against harmful effect of gastric and duodenal environment (Siro et al. 2008).

Grzeskowiak et al. (2011) have demonstrated that different isolates of the same strain (*L. rhamnosus* GG) had different properties that could influence their *in vivo* effects. This study emphasized the importance of controlling the manufacturing process and the food matrix since previous studies have indicated that the vehicle could affect the strain properties (Kankaanpaa et al. 2001; Kankaanpaa et al. 2004). Moreover, in a recent review, they reported that some studies have shown that a probiotic mixture was not more effective than a single strain. The hypothesis is that a greater variety of strains reduce the effectiveness of a multi-strain probiotic. The many species could inhibit each other by production of antagonistic agents or by competition for the nutrients or binding sites in the GI tract (Chapman et al. 2011). Therefore, it is primordial not only to choose strains that coexist, but also act synergistically. This, combine with the manufacturing process and individual variability, could explain the different results obtained between the probiotic products evaluated in this study.

Millette *et al.* (Millette et al. 2008) demonstrated that the probiotic mixture of *L. acidophilus* CL1285 and *L. casei* LBC80R could resist the gastric conditions at pH ≥ 2.5, which is consistent with the findings in this study. For the probiotic strain, *L. rhamnosus* GG, large losses (up to 6 log) were observed with the addition of bile salts in another study (Sumeri et al. 2008). These results confirm those of this study because the probiotic capsule #13 contained only *L. rhamnosus* GG and its initial count was 10.06 log CFU/g with a final count lower than 3.8 log CFU/g after the intestinal treatment, which is a loss of more than 6 log. Clinical studies also demonstrated that *L. casei* DN-114 001 could survive the GI tract in infants and adults (Oozeer et al. 2006; Tormo Carnicer et al. 2006). This effect was confirmed in this study with the #19 having a good survival rate. Favaro-Trindade and Grosso (2002) showed that free *L. acidophilus* La-05 and *B. lactis* Bb-12 were tolerant to bile acid *in vitro* even when the concentration was greater than the normal concentration found in the human intestine. Moreover, these strains underwent a slight reduction of concentration at pH 2, but were completely destroyed at pH 1 after one hour. In this study, the probiotic capsules #17 was not able to survive the gastric conditions at pH 1.5 and the intestinal conditions.

In conclusion, our study showed the importance of evaluating the survival of probiotic strains in the finished product since their viability could be modified during the manufacturing process. It also showed that all probiotic products were not similar and that some could not even survive the harsh environment of the GI tract in order to exert their beneficial effects. Therefore, because we observed that the majority of the probiotic products have failed to protect the GI survival of the strains, it would be important for manufacturers to develop technologies to ensure this ability. quality and the efficacy of the products. Finally, the use of enteric coating of encapsulated probiotic bacteria seem to be effective to preserve bacterial viability during the GI passage.

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