Food Research International 41 (2008) 539–543

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Effect of storage in a fruit drink on subsequent survival of probiotic lactobacilli to gastro-intestinal stresses

Claude P. Champagne *, Nancy J. Gardner [1]

Food Research and Development Center, Agriculture and Agri-Food Canada, 3600 Casavant, St-Hyacinthe QC, Canada J2S 8E3

ARTICLE INFO

Article history:
Received 20 November 2007
Accepted 3 March 2008

Keywords:
Lactobacillus
pH
Fruit drink

ABSTRACT

Nine probiotic lactobacilli strains were evaluated for their ability to survive in a commercial fruit drink stored at 4 °C for up to 80 days. The pH of the drink was 4.2, which enabled good stability of many cultures during storage. *Lactobacillus rhamnosus* seemed more stable than *Lactobacillus acidophilus* in this medium, but viability was still mostly strain dependent. Spectrophotometry studies showed that the cultures generally had higher growth rates as pH increased from 3.8 to 4.2. There was a correlation ($P = 0.037$) between stability during storage in the drink and the ability of the strains to grow at pH 4.2, but that the relationship was not strong ($R^2 = 0.49$). Four strains were selected to study their resistance to simulated gastro-intestinal (GI) conditions. Fresh cultures were compared to those obtained after 35 days of storage at 4 °C. Viability in presence of 0.3% bile salts or of pancreatic enzymes was not affected by previous refrigerated storage. However, the cultures which were tested after having been stored for 35 days at 4 °C in the fruit drink had on the average 1.2 log higher viability losses than the fresh cultures when exposed to a 2 h incubation at pH 2.0 to simulate a gastric stress.

1. Introduction

Probiotics can be defined as "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Araya et al., 2002). Those health-promoting microorganisms have traditionally been included into commercial dairy products (Leatherhead, 2006; Shin, Lee, Pestka & Ustunol, 2000). Even though fermented dairy products are generally good matrices for the delivery of probiotics to human (Sarrela, Mogensen, Fonden, Matto, & Mattila-Sandholm, 2000), other foods have been examined for their potential as probiotic carriers. Mayonnaise, soymilk, meats, baby foods, ice creams, fruit drinks and many others have been proposed (Champagne, Roy, & Gardner, 2005).

Fruit drinks could serve as good probiotic carriers if precautions are taken in regards with sensory characteristics (Luckow & Delahunty, 2004) and pH. With respect to pH, probiotic bacteria loose viability during storage in many fermented milks having pH values between 4.0 and 5.0 (Champagne et al., 2005). Therefore, the even more acid environment of some fruit juices, having pH values around 3.5, is of concern (Sheehan, Ross, & Fitzgerald, 2007). One method of raising pH in a fruit juice is to blend in milk ingredients. Data on stability of probiotics in fruit juices have mostly been obtained in pure juices (Parish, Sadler, & Wicker, 1990; Saarela, Virkajarvi, Nohynek, Vaari, & Matto, 2006; Sheehan et al., 2007). However, little is known of the stability of probiotics in blends designed to have pH values above 4.0. This was the first aim of this study.

Although strain selection for addition to foods must be based on the sensory impact that the culture has on the final product (Luckow, Sheehan, & Fitzgerald, 2005) stability during storage and health benefits are of primary importance. Although there are instances where non-viable cells have shown health benefits (Ouwehand & Salminen, 1998) it is still considered desirable to have live cultures in the products (Stanton, Ross, Fitzgerald, & Sinderen, 2005). Probiotics should not only survive in the food product but cells should also be able to reach the small intestine alive. It has been demonstrated that the food matrix affects survival to the gastric environment (Stanton et al., 1998; Saarela, Virkajarvi, Alakomi, Sigvard-Mattila, & Matto, 2006), but it is unknown if storage of probiotic bacteria in a fruit juice affects their subsequent ability survive the gastro-intestinal stresses brought by acid, bile and enzymes. This was the second aim of this study.

In this study we report of the stability to storage at 4 °C of 9 probiotic lactobacilli in a drink composed of 10 fruit and dairy ingredients, as well as the ability of the cultures to survive simulated gastro-intestinal stress conditions following storage.

* Corresponding author. Tel.: +1 450 768 3238.
E-mail address: ChampagneC@agr.gc.ca (C.P. Champagne).
[1] Present address: Canadian Food Inspection Agency, 3400 Casavant, St-Hyacinthe QC, Canada J2S 8E3.

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doi:10.1016/j.foodres.2008.03.003

2. Materials and methods

2.1. Bacterial strains and growth conditions

Probiotic strains were from the Food Research and Development Center culture collection, Institut Rosell (Lallemand Inc.; Montreal, QC, Canada), Chr. Hansen (Hørsholm, Denmark), Probi SA (Lund, Sweden) or the American Type Culture Collection (ATCC; Rockport, MD, USA). In order to prevent any commercial prejudice to the companies, they were given internal codes, ranging from LB2 to LB42. The strains were thus *Lactobacillus acidophilus* LB2, LB3 and LB45, *Lactobacillus brevis* LB6, *Lactobacillus rhamnosus* LB11 and LB24, *Lactobacillus fermentum* LB32, *Lactobacillus plantarum* LB42 and *Lactobacillus reuteri* LB38. The freeze-dried cultures were rehydrated 10 min at room temperature in a medium composed of 1.5% peptone (Difco – Becton Dickinson; Sparks, MD, USA), 1.0% tryptone (Difco) and 0.5% yeast extract (Difco). The rehydrated culture (1 mL) was inoculated into 100 mL of MRS broth (Difco) and incubated at 37 °C for 16 h. Stock cultures were prepared by mixing the fresh MRS-grown culture with 20% (w/w) sterile rehydrated skim milk and 20% sterile glycerol in a 1:2:2 ratio and placing 1 mL fraction in sterile cryogenic vials (Corning Inc.; Corning, NY, USA). The cell suspensions were then frozen at –70 °C until used.

For the preparation of the cultures used in automated spectrophotometry (AS) assays and the survival assays in fruit juices, one vial was thawed at room temperature and used immediately to inoculate 100 mL of MRS broth. After incubation at 37 °C until a pH of 4.8 was obtained, 10 mL of the culture was centrifuged at 3000 g for 20 min at 4 °C, and the cell pellet was suspended in 10 mL of sterile saline 0.9% (w/v).

2.2. Survival in fruit juice

A series of 400 mL portions of the commercial beverage Oasis Health Break™ (Lassonde Inc., Rougemont, QC, Canada) were dispensed in sterile glass bottles. The ingredients in the fruit juice drink were, per order of importance: fruit juice concentrates (pineapple, apple, orange, pear and/or grape, passion fruit, lemon), purees (peach, strawberry, mango and kiwi), yoghurt powder (dairy ingredients, lactic acid, citric acid, bacterial cultures), modified dairy ingredients, natural flavours, pectin, guar gum, ascorbic acid and beta-carotene. The products had been heat treated under the Oasis commercial conditions prior to shipping to the Food Research and Development Centre (FRDC). The juice beverages were then inoculated with 4 mL of fresh cell suspension of lactobacilli. The inoculated juices were then stored at 4 °C under aerobic conditions, and special care taken to maintain that temperature during sampling. Viable counts and pH analyses were carried out at weeks 1–5, as well as after 80 days of incubation. Three independent replications were conducted.

Viable cell counts were obtained by plating the appropriate 0.1% peptone dilution in MRS (Difco) agar. Lactic cultures tend to produce cell chains, and a high-shear agitation step was included in the methodology in order to break them as much as possible. Thus, in the first dilution tube, a 30 s homogenisation step was carried out using sterile disposable Omni-tips generator probes on an Omni TH (Omni International, USA) unit operated at maximum speed. Viable counts were obtained using the pour plate method on Difco MRS Agar (Becton Dickinson, Sparks, MD, USA). The plates are incubated at 37 °C for 48 h under aerobic conditions.

2.3. Effect of pH on biomass levels

The nine lactobacilli were examined for their ability to survive or grow under acidic conditions with AS. The juice blend had turbidity, which precluded its use in AS. Therefore, cultures were prepared in a basal medium (BM) composed per litre: 20 g glucose, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot H_2O$, 3 g K_2HPO_4, 3 g KH_2PO_4, 3 g CH_3COONa, 3 g yeast extract (Difco) and 1 g Tween 80. The pH was adjusted with HCl 6 N to obtain final values of 4.2, 4.0, 3.8, or 3.6 prior to sterilisation by filtration (0.22 μm pore membrane; Millipore, USA). A high pH control medium was prepared at pH 6.0. The basal medium (245 μL) was dispensed in the microplate wells (96 wells, Costar) and inoculated with 5 μL of fresh saline cell suspension. A well of each medium was kept uninoculated in order to estimate the baseline optical density (OD) of each condition. The plates were then placed into the Powerwave X incubation chamber (BioTek Instruments; Winooski, VT, USA), and shaking frequency (10 s every 15 min), shaking intensity (medium), temperature (37 °C) OD wavelength (600 nm) and OD reading frequency (every 10 min) were set. Three wells per strain for each condition were prepared in each assay, and three independent repetitions were carried out.

2.4. Survival in the simulated gastro-intestinal conditions

After 35 days of storage at 4 °C, 4 of the 9 cultures were evaluated for their survival to the exposure to simulated gastro-intestinal stresses. They were compared with a freshly prepared culture. Three *in vitro* treatments were used to simulate digestive stresses, which were adapted from Charteris, O'Neill and Kelly (1994). BM was prepared as described previously and was used as the base medium for three analyses: effect of low pH, effect of pancreatic enzymes and effect of bile salts.

2.4.1. Stability at pH 2.0

10 mL of BM medium was adjusted at pH 2.0 after sterilisation by the addition of 145 μL of a 6 N HCl solution.

2.4.2. Effect of pancreatic enzymes

BM was supplemented with a 50 mg/mL pancreatin solution (Sigma), which had previously been centrifuged at 2000*g* for 15 min at 4 °C and sterilized by filtration with a 0.22 μm membrane; 380 μL of the enzyme solution was then aseptically added to 10 mL of the BM to a final pancreatin concentration of 1.9 mg mL^{-1}. The pH was set at 6.0.

2.4.3. BM with bile salts

0.3% of bovine Oxgall was added to BM, pH was adjusted to 6.0, and sterilisation by filtration was then carried out. BM at pH 6.0 was used as a control.

The BM were brought to 37 °C and then inoculated with 10% (vol/vol) of the probiotic-containing fruit beverage and incubated 2 hours. Cell counts were evaluated on MRS agar as described previously. Three independent assays were carried out for each simulated gastro-intestinal stress. The "fresh culture" control was prepared by centrifuging a freshly-grown MRS culture at 3000 × g, and adding the fruit juice blend to the cell pellet in order to obtain approximately 10^7 cfu mL^{-1}. In all three simulated GI conditions, three independent assays were carried out.

3. Results and discussion

3.1. Stability during storage in the fruit juice blend

The two current probiotic-carrying fruit juice blends on the Canadian market contain between 1 and 3 billion probiotic cells per 250 mL portion. Therefore, inoculation by approximately 10^7 cfu mL^{-1} is a commercially representative level of juice supplementation, and assays were carried out to obtain this addition le-

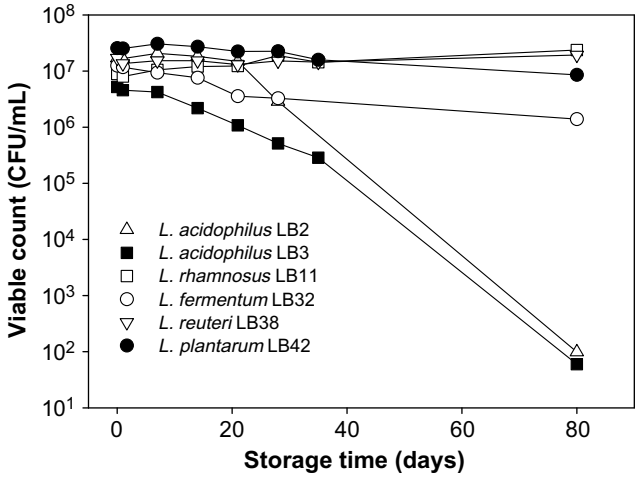

Fig. 1. Viability of various probiotic cultures in a fruit juice blend during storage at 4 °C for up to 80 days.

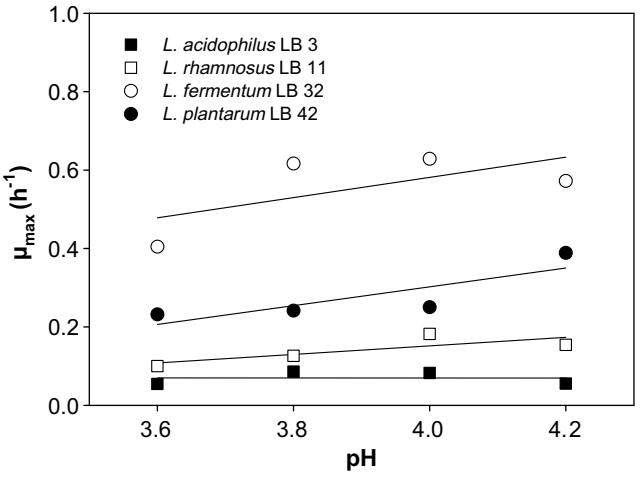

Fig. 2. Effect of pH on maximum growth rates (μ_{max}) of lactobacilli in the base medium (BM).

vel. Slight variations in inoculation levels occurred due to different populations reached in the MRS broth prior to centrifugation.

The pH of the juice remained unchanged after 28 days of storage indicating weak metabolic activity of the probiotic at 4 °C. Most strains still had high viabilities after 80 days of storage at 4 °C (Fig. 1). For reasons of clarity, not all culture viability curves were presented on Fig. 1 because of similarities. Thus, *Lactobacillus brevis* LB6, *L. rhamnosus* LB24 and *L. acidophilus* LB45 had similar patterns to *L. fermentum* LB32, *L. reuteri* LB38 and *L. plantarum* LB42, respectively. Two *L. acidophilus* cultures, LB2 and LB3, had the lowest stability during storage. In this study, both strains of *L. rhamnosus* showed good viability during storage, which was in line with data of Sheehan et al. (2007) in orange and pineapple juices. However, much higher losses in *L. rhamnosus* viability were reported in apple juice (Saarela et al., 2006). Data from this study point to greater losses in viability of *L. acidophilus* than of *L. rhamnosus* (Fig. 1) to storage in acid foods, which is line with the literature (Garro, de Valdez, Oliver, & de Giori, 1999; Tharmaraj & Shah, 2004). However, this only applied to two of the 3 strains of *L. acidophilus*.

It is known that probiotics loose viability during storage in acid environments (Dave & Shah, 1997; Sheehan et al., 2007) or in the presence of oxygen (Talwalkar & Kailasapathy, 2004). With the methodology used in this study, the plating procedure for the viable counts was carried out aerobically. Fresh cultures gave good results in this situation. However, there is a possibility that viable but acid or oxygen-stressed cells obtained at the end of the storage period may not have generated colonies and that the methodology used under-estimated viable counts. This should be kept in mind while interpreting the viability data. Additionally, the food formulation strongly affects the viability of probiotics during storage (Mattila-Sandholm, Myllarinen, Crittenden, Mogensen, Fonden, & Sareela, 2002; Saarela et al., 2006). Fruit juices may contain natural microbial growth inhibitors or additives, such as colorings and flavourings, which can be associated with loss of viability (Vinderola, Costa, Regenhardt, & Reinheimer, 2002). Therefore, variations in strain stability observed in this study may be due to pH, juice composition or oxygen. It was therefore examined to what extent the pH itself affected the cultures.

High temperature incubations have been proposed in attepts to forecast the stability of microbial cultures during storage (King, Lin, & Liu, 1998; Mitic & Otenhajmer, 1974). High temperature incubation is also done to evaluate the stability of foods and basically consists of an accelerated storage test. The cultures were therefore incubated at 37 °C, and AS used to follow the evolution of biomass. A loss of viability with subsequent autolysis would have resulted in a drop of OD (Thiboutot, Dako, El Soda, Vuillemard, Power, & Simard, 1995) and have generated negative μ values. This was not observed for any of the strains. Rather, constant or increasing OD values were recorded. In this accelerated pH effect test, AS showed that the cultures generally had higher growth rates as pH increased from 3.8 to 4.2 (Fig. 2). Strains LB6, LB24, LB38 and LB 45 were not presented on Fig. 2 because they had patterns which fell between those of *L. rhamnosus* LB 11 and *L. plantarum* LB42. *Lactobacillus acidophilus* LB2 had the same pattern as that of *L. acidophilus* LB3, and showed the lowest growth rates of all strains.

The low stability during storage of *L. acidophilus* LB2 and LB3 (Fig. 1) and their low μ_{max} values at acid pH values suggested that there was a link between these two parameters. Indeed, Sheehan et al. (2007) have shown that pH values below 4.5 were detrimental to storage stability of probiotic bacteria. It cannot be automatically assumed that phenomena which occur at high temperatures will extend to lower incubation temperatures. A regression analysis carried out between storage losses in viability (log values) and the μ_{max} values at pH 4.2 showed that there was a statistically significant correlation ($P = 0.037$), but that the relationship was not strong ($R^2 = 0.49$). This shows that factors which affect growth at optimum temperatures do partially apply to stability at refrigerated temperatures. Since lower pH conditions resulted in lower μ_{max} values (Fig. 1) it can be hypothesized that the pH of the medium constituted one of the factors which explain the stability of various strains during storage in juice. Thus, the good stability of many strains in this particular fruit juice drink was partially linked to the selection of a pH = 4.2 blend which incorporated dairy ingredients.

3.2. Viability during simulated gastro-intestinal stresses

Four strains were selected to study their resistance to simulated gastro-intestinal (GI) conditions, mainly based on variable stability during storage. Indeed, *L. acidophilus* LB3 and *L. rhamnosus* LB11 were respectively the least and most stable cultures. Furthermore, *L. reuteri* was of interest because it is considered to be of the few true indigenous *Lactobacillus* species in humans (Valeur, Engel, Carbajal, Connolly, & Ladefoged, 2004) while *L. plantarum* is rather associated with the fermentation of vegetal substrates (Alwazeer, Cachon, & Divies, 2002).

Table 1
Effect of storage in a fruit juice blend for 35 days at 4 °C on subsequent viability losses of 4 probiotic cultures to 2 h of incubation at 37 °C in conditions simulating gastro-intestinal stresses

Culture condition	Strain	Viability loss (log CFU/mL) after treatment[a]		
		Acid (pH 2)	Bile (0.3%)	Pancreatic enzymes
Fresh culture	*L. acidophilus* LB3	2.8	1.2	0
	L. rhamnosus LB11	2.8	0.3	0
	L. reuteri LB38	2.7	0.1	0
	L. plantarum LB42	2.6	0.2	0
Stored (35 days)	*L. acidophilus* LB3	5.0	0	0
	L. rhamnosus LB11	4.5	0	0.1
	L. reuteri LB38	3.2	0	0.2
	L. plantarum LB42	3.7	0	0

[a] In reference to control treatment (Base medium at pH 6.0).

None of the strains were significantly affected by the incubation in presence of 0.3% bile salts or the pancreatic enzymes (Table 1). Storage for 35 days in the fruit juice blend did not affect the strains sensitivity to these compounds. In probiotic selection, tolerance to the environment of the small intestine is also thought to be of importance since the acid-sensitive strains can be buffered through the stomach (Huang & Adams, 2004; Leverrier, Fremont, Rouault, Boyaval, & Jan, 2005). Data from this study suggest that a 35 days storage in the fruit drink would not affect sensitivity of probiotics to bile or pancreatic enzymes.

The literature shows that a low final pH during bacterial growth induces an acid tolerance response (Lorca, & Font de Valdez, 2001; Hartke, Bouché, Giard, Benachour, Boutibonnes, & Auffray, 1996). The induction of this pH stress response may protect probiotic bacteria not only from acid challenge but also from other stresses such as heat, osmotic or oxidative shocks (Van de Guchte, Chervaux, Smokvina, Ehrlich, & Maguin, 2002). It was therefore hypothesized that cells which were stored in the acid fruit drink could have enhanced resistance to the subsequent acid challenge of the simulated GI stress. The opposite was observed. There were strong viability losses following a 2 h/37 °C incubation at pH 2.0 (Table 1). Furthermore, the cultures which were tested after having been stored for 35 days at 4 °C in the fruit drink had on the average 1.2 log higher viability losses than the fresh cultures, and paired *t*-tests showed that this difference was statistically significant ($P = 0.034$). In this study, the fresh cell cultures were harvested during their stationary phase following a fermentation without pH control and cultures had pH values below 5.0. Therefore, exposure to acid to induce the acid stress response was carried out for both sets of cultures. Evidently, a short-time exposure of the cultures to acid at 37 °C provides better protection to subsequent GI conditions than does an extended low-temperature storage period.

Since the culture which had the lowest stability during storage in the juice blend (*L. acidophilus* LB3) also had the highest viability losses in the simulated GI acid test, it was examined if a direct relationship existed between the two sets of data. The correlation ($R^2 = 0.50$) was not strong and was not judged to be statistically significant ($P = 0.2$) due to the limited number of experimental data (only 4 strains). Corcoran, Stanton, Fitzgerald, & Ross, 2005) found that glucose enhanced the survival of lactobacilli in acidic conditions. Therefore, in order to better represent actual GI conditions, and prevent discrepancies between viability loses during storage and in the GI stress tests, the simulated GI procedure used in this study was designed to incorporate a carbohydrate as well as food components. This being stated, this classical simulated GI acid procedure at pH levels between 1.5 and 2.5 probably overestimate actual gastric viability losses due to the buffering ability of certain foods (Mainville, Arcand, & Farnworth, 2005).

4. Conclusion

These data have two impacts of the food industry. First, while data from this study add to the literature with respect stability of probiotics in fruit juices, results also point to strong variability in stability between species and strains. Companies wishing to develop new products unfortunately need to carry out tests with the strains they have chosen. Secondly, stored cultures seem to be less resistant than fresh cells to an acid challenge as would occur in the stomach following consumption of the products. This was observed with probiotic bacteria stored in a fruit juice, but raises a concern with respect to other acid foods such as yoghurt. More research is warranted on the effect of storage on the functional properties of probiotics. It remains to be determined to what extent the age of the stored cultures influence their demonstrated clinical health benefits.

Acknowledgments

The authors wish to express gratitude to Natacha Salehi for technical work as well as to Sasithorn Tajchakavit and Lassonde Inc. for providing the commercial fruit juice blends.

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