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Protection of *Bifidobacterium lactis* and *Lactobacillus acidophilus* by microencapsulation using spray-chilling

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ABSTRACT

The aim of this study was to produce and evaluate solid lipid microparticles containing *Bifidobacterium lactis* or *Lactobacillus acidophilus*. Survival assays were conducted to evaluate the resistance of the probiotics to spray-chilling process, their resistance to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) and their stability during 90 d of storage. The viability of the cells was not affected by microencapsulation. The free and encapsulated cells of *B. lactis* were resistant to SGF and SIF. The microencapsulation, however, provided protection for *L. acidophilus* against SGF and SIF. The free and encapsulated microorganisms lost their viability when they were stored at 37 °C. However, promising results were obtained when refrigerated and frozen storage was applied. The study indicates that spray-chilling using fat as carrier can be considered an innovative technology and matrix, respectively, for the protection, application and delivery of probiotics.

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1. Introduction

Over the last twenty years, there has been an increased interest in the role of probiotic bacteria in human health (Chandramouli, Kailasapathy, Peiris, & Jones, 2004). Probiotics are living microorganisms which, when administered in adequate levels, confer health benefits to the host (FAO/WHO, 2006). *Bifidobacterium lactis* and *Lactobacillus acidophilus* are probiotic microorganisms that are frequently used in food products. However, these organisms must remain viable during the entire shelf life of the product, and they must pass through the stomach and intestine to provide beneficial effects on human health (Laroia & Martin, 1990). Because these microorganisms are sensitive to a series of factors, which include especially the presence of oxygen and acidic media, microencapsulation has been studied as an alternative to maintain the viability of the probiotic cells (Fung, Yuen, & Liong, 2011; Kim, Kamara, Good, & Enders, 1988; Kwok, Groves, & Burgess, 1992; Nag, Han, & Singh, 2011; Oliveira et al., 2007a, 2007b).

To date, novel microencapsulation technologies and shell materials have been investigated for improving the viability of probiotic bacterial cells at low pH values and high bile concentrations (Lisboa, Andrade, & Oliveira, 2009) and cell stability over storage (Heidebach, Först, & Kulozik, 2010; Rodrigues et al., 2011). Among all the techniques that have been used to encapsulate probiotics, the following should be highlighted: extrusion, atomisation or spray drying, emulsion, coacervation and immobilisation in fat and starch granules (Favaro-Trindade, Heinemann, & Pedroso, 2011).

Lahtinen, Ouwehand, Salminen, Forssell, and Myllärinen (2007) immobilised bifidobacteria in a lipid matrix that was based on cocoa butter. This process resulted in the increased viability of the cells during storage in model systems that simulated fermented and unfermented beverages. This result suggests that the lipid matrix protects cells against storage stress, and it possibly blocks them from exposure to water and stressors, such as H^+ ions. Therefore, the incorporation of probiotics into lipid microparticles is an extremely interesting approach for food products. Furthermore, due to the composition of lipid materials, an advantage of these microparticles is that they are easily digested in the intestine by lipases, which causes the microorganisms to be released in the vicinities of their site of action (Favaro-Trindade et al., 2011).

Among the various techniques that are used for microencapsulation, spray-chilling (also called spray cooling and spray congealing) can use lipids as wall materials. This process is similar to spray drying with respect to the production of fine droplets. However, spray-chilling is based on the injection of cold air, which enables the solidification of the particle; a molten matrix that contains the bioactive compound is atomised so that it forms drops that quickly solidify when they contact the cold air (Champagne & Fustier, 2007). The spray-chilling process is typically referred to as 'matrix'

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encapsulation in the literature, in which the active ingredient particles are buried in the fat matrix. However, a significant proportion of the active ingredient exists on the surface of the microparticles or protrudes from the fat matrix, where it can directly access the environment (Gouin, 2004). The microparticles that are produced can present some disadvantages, which include a low encapsulation capacity and the expulsion of core material during storage, due to the crystalline structure and polymorphic arrangement characteristic of many lipid materials during the solidification and crystallisation process (Sato & Ueno, 2005; Westesen, Bunjes, & Koch, 1997). However, spray-chilling is considered to be the cheapest encapsulation technology that has the possibility of industrial scale manufacture (Gouin, 2004; Sato & Ueno, 2005; Westesen et al., 1997). According to Champagne and Fustier (2007), spray-chilling deserves greater attention for the microencapsulation of probiotics because it may expand the range of matrices that are used. Moreover, this technology could be used to generate smaller beads, which may be desirable in food processing.

To date, spray-chilling has been applied for conserving enzymes, flavours, minerals and proteins (de Vo, Faas, Spasojevic, & Sikkema, 2010). However, complete studies on the use of the spray-chilling technique on the microencapsulation of probiotics have not been found in the scientific literature. Therefore, the aim of this study was to produce and evaluate the solid lipid microparticles of *B. lactis* and *L. acidophilus* that were produced by spray-chilling technology using a special vegetable fat as the carrier.

2. Materials and methods

2.1. Carrier

An interesterified fat with palm and palm kernel, which was kindly donated by Vigor (São Caetano do Sul, Brazil), was used as a meltable carrier or encapsulating agent. Its melting point is 47.5 °C.

2.2. Culture preparation

B. lactis (BI-01) and *L. acidophilus* (LAC-04), which were kindly donated by Danisco (Cotia, Brazil), were activated as described by Liserre, Ré, and Franco (2007) with some modifications. The freeze-dried cultures were activated in reconstituted skim milk (100 g L^{-1}) that contained 1% (w/v) glucose, 1% (w/v) sodium citrate, 1% (w/v) yeast extract and 0.05% (w/v) cysteine for 20 h at 37 °C. The cells were harvested by centrifugation at 2360 × *g* for 8 min and washed twice in NaCl solution (0.85% w/v). The pellet was resuspended in the saline solution to obtain a suspension that contained approximately 10^9–10^{10} cfu mL^{-1}. The suspension was maintained in a glass vessel with cover at −18, 7 and 37 °C for the future assays of resistance and viability.

2.3. Enumeration of probiotic cells

The viable *B. lactis* and *L. acidophilus* cells were counted by the pour-plate technique on man, rogosa and sharpe (MRS) agar according to Grosso and Fávaro-Trindade (2004) with some modifications. For *B. lactis*, MRS agar was supplemented with lithium chloride (0.1%), L-cysteine (0.05%) and aniline blue (0.01%). Serial dilutions were prepared with a 2% sodium citrate solution. The plates were incubated in anaerobiosis with the anaerobic system (Probac, São Paulo, Brazil) at 37 °C for 72 h. The plating was performed in duplicate. For the enumeration of probiotics in the microparticles, however, a pre-heated sodium citrate solution at 48 °C was used to melt the fat matrix and promote the release of the cells during serial dilutions.

2.4. Preparation of microparticles

The microparticles were prepared with a spray chiller (Labmaq, Sertãozinho, Brazil) according to the method described by Chambi, Alvim, Barrera-Arellano, and Grosso (2008) with some modifications. A solution that contained molten fat at 48 °C, probiotics and lecithin (1%, as a surfactant) was homogenised using an Ultraturrax (IKA, Staufen, Germany) at 9500 rpm for 60 s. The proportions of reactivated cells: fat of 1:10 (*B. lactis*) and 1:4 (*L. acidophilus*) were used to obtain microparticles that contained levels of viable cells of at least 10^7 cfu g^{-1}. The lipid microparticles were then formed by the atomisation of the emulsion into a cold chamber at 10 °C using a double-fluid (∅ = 0.7 mm) atomiser with an atomisation air pressure of 0.98 kPa. The microparticles were maintained in a glass vessel with cover at −18 °C for the future assays of resistance, viability, and characterisation. The microparticles were also maintained at 7 °C and 37 °C for the shelf life assays. The encapsulation process and all analyses were conducted in duplicate.

To determine the effect of spray-chilling on the viabilities of the microorganisms, cell counts were performed before the microencapsulation process (at the emulsion step) and in the microparticles.

2.5. Characterisation of microparticles

Microparticle morphology was evaluated using a scanning electron microscope (JSM; Jeol, Tokyo, Japan) according to Oliveira et al. (2007a), and optical microscope (Bel, Monza, Italy) using a digital camera and an image program (Bel MicroImage Analyzer, Monza, Italy). The particle size distribution of the microparticles was measured using the laser-diffraction technique by Shimadzu Sald-201V (Tokyo, Japan), using ethanol as the dispersion medium.

2.6. Evaluation of the resistance of B. lactis and L. acidophilus to simulated gastric and intestinal fluids and of the stability of the microencapsulated microorganisms during storage

The resistance to the in vitro simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was determined according to the method described by Gbassi, Vandamme, Ennahar, and Marchioni (2009). Free and encapsulated cells were incubated in SGF for 120 min and in SIF for 180 min at 37 °C. SGF consisted of 9 g L^{-1} of sodium chloride (Synth, Diadema, Brazil) and 3 g L^{-1} of pepsin from porcine stomach mucosa (Sigma–Aldrich, St. Louis, MO, USA), and the pH was adjusted to 1.8 with hydrochloric acid (Synth, Diadema, Brazil). SIF consisted of 9 g L^{-1} of sodium chloride, 10 g L^{-1} each of pancreatin and trypsin from bovine pancreas (Sigma–Aldrich, St. Louis, MO, USA) and 3 g L^{-1} of bile salts (Oxgall, Difco, Hampshire, UK), and the pH was adjusted to 6.5 with sodium hydroxide (Synth, Diadema, Brazil). Survey assays were conducted at 0, 60 and 120 min (SGF), and 0, 90 and 180 min (SIF) of incubation.

The evaluation of the stability of the microencapsulated microorganisms during storage was designed to determine the shelf life of the obtained microparticles. The number of viable cells was counted after 1, 30, 60 and 90 d of storage at −18 °C, 7 °C and 37 °C (Oliveira et al., 2007a).

3. Results and discussion

3.1. Morphological characterisation and mean diameter of the microparticles

After spray-chilling, a solidified, white and cooled free-flowing powder was collected.

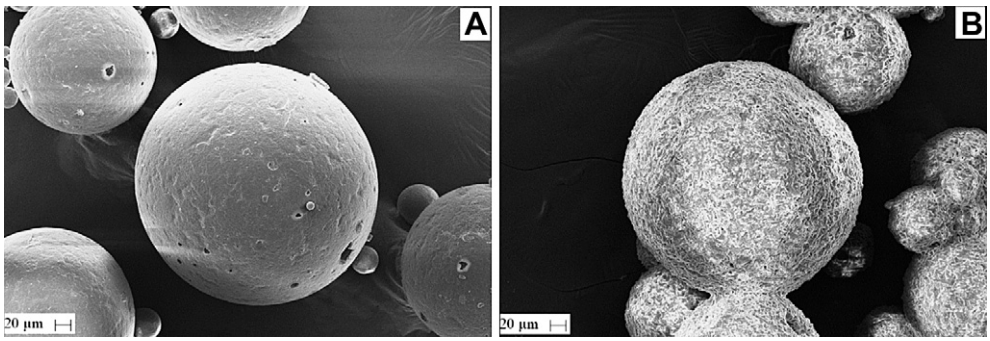

Fig. 1. Scanning electron microscopy images of the microparticles containing *B. lactis* (A) and *L. acidophilus* (B) prepared by spray-chilling (500× magnification).

The scanning electron micrographs (Fig. 1) revealed that the process of spray-chilling produced microparticles that displayed spherical shapes; this result is interesting because this format facilitates the flow of material, although it is not only shape that determines flowability. This shape was also observed by Chambi et al. (2008) and Savolainen, Khoo, Glad, Dahlqvist, and Uppo (2002) when they encapsulated drugs and water-soluble compounds by spray-chilling using stearic acid and a mixture of

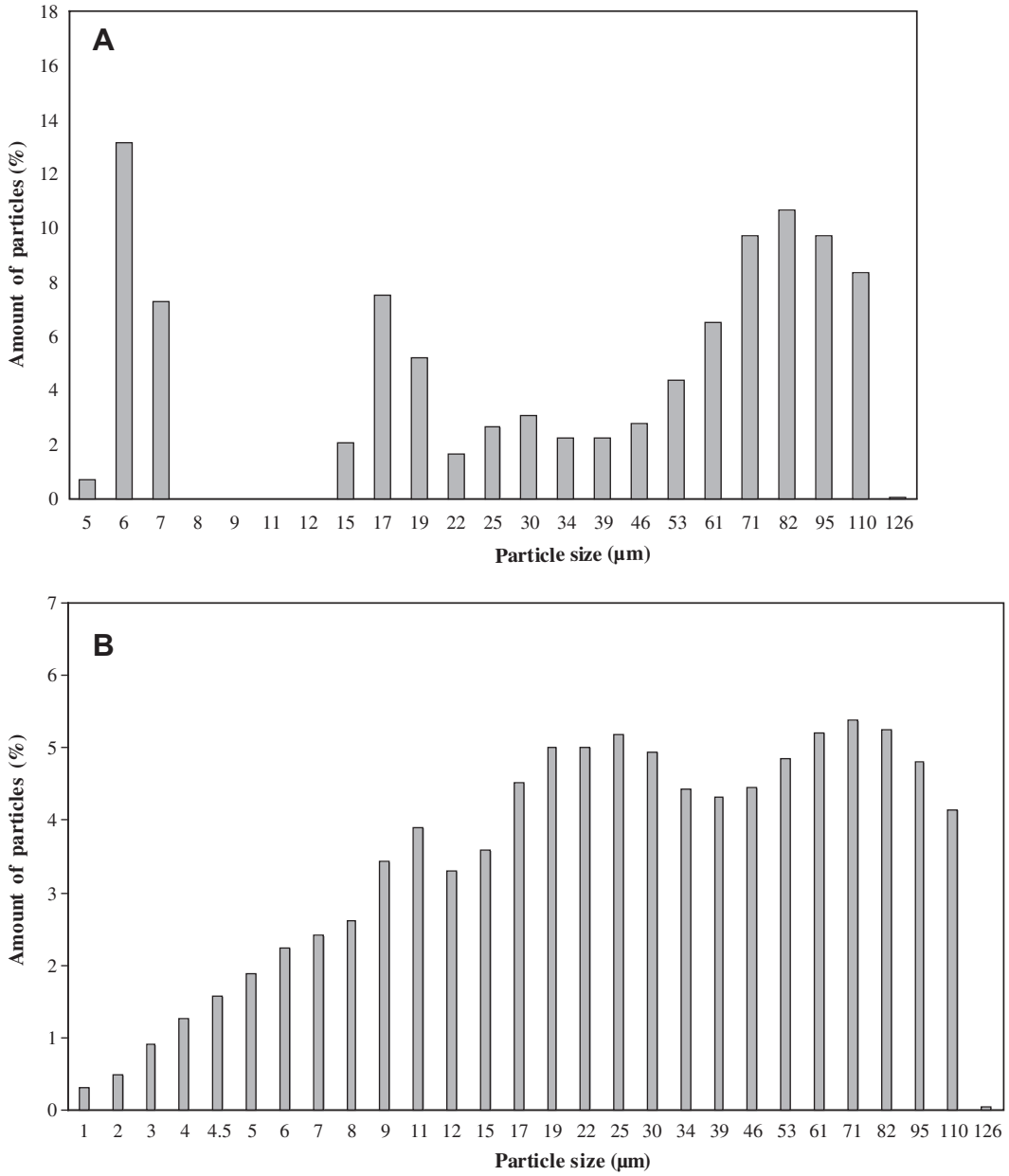

Fig. 2. Particle size distribution of the microparticles containing *B. lactis* (A) and *L. acidophilus* (B).

stearic acid, lauric acid and sorbitan tristearate as the encapsulating agents, respectively. In addition, the photomicrography revealed that although the microparticles were spherical in shape, the walls of the particles were irregular and quite wrinkled. In addition, as seen in Fig. 1B, an increased aqueous portion in the emulsion, such as in the *L. acidophilus* particles, correlated with an increased presence of wrinkles. The *B. lactis* and *L. acidophilus* cells were not visible on the surface of the microparticles by scanning electron microscopy (SEM) photomicrography, even at a high magnification (5000×).

The microparticle size is an important characteristic for food application purposes because a larger particle size correlates with an increasingly detrimental effect on the food texture. Considering alginate beads, Hansen, Allan-Wojtas, Jin, and Paulson (2002) reported that a desirable size is approximately 100 μm. A laser-diffraction technique confirmed the presence of different particle sizes that were smaller than 100 μm, however. The mean diameter of the *B. lactis* microparticles was 40.3 μm, and the particle size ranged from 0.9 to 126.9 μm (Fig. 2A); the mean diameter of the *L. acidophilus* particles was 24.0 μm, and the size ranged from 0.6 to 126.9 μm (Fig. 2B).

3.2. *Viability of* B. lactis *and* L. acidophilus *due the microencapsulation process*

Because the microencapsulation of probiotic microorganisms is designed to increase the stability and viability of the probiotic culture in several types of products during processing and storage, and in the presence of gastric and intestinal fluids, the choice of the encapsulation method is critical (Favaro-Trindade & Grosso, 2000, 2002; Oliveira et al., 2007b). The techniques most commonly used in the microencapsulation of probiotics are emulsion, extrusion and spray drying (Favaro-Trindade et al., 2011). Spray-chilling is an alternative process for the protection of probiotic cultures, which facilitates the application of probiotics and could expand their uses. However, little is known about the microencapsulation effects on the viability of the cells and the protection of probiotics against external agents. Table 1 presents the counts in the emulsion and in the microcapsules. Minimal differences were observed due to processing. The results obtained in the present study suggest that spray-chilling technology was an efficient microencapsulation process because the viability of *B. lactis* and *L. acidophilus* was not affected. This result revealed that the conditions employed in the process, such as atomisation and chilling, were sufficiently mild towards the microorganisms under study, and they guaranteed the cellular integrity of the majority of the population. It is unknown, however, if the homogenisation process or the emulsifier used to create the emulsion affected viable counts.

3.3. *Resistance of* B. lactis *and* L. acidophilus *to the simulated gastric and intestinal fluids*

Before *B. lactis* and *L. acidophilus* can be used as functional products, they must survive until they reach the gastrointestinal tract, pass through it, and colonise the intestine (Kim et al., 1988). However, one of the major problems in the efficacy of probiotic foods is the low survival rate of the microorganisms in gastric pH

Table 1
Resistance of *B. lactis* and *L. acidophilus* to spray-chilling process.[a]

Microorganism	Viable cells (count in $\log_{10}$ cfu g^{-1})	
	Emulsion	Microparticle
B. lactis	8.2 ± 0.1	8.3 ± 0.1
L. acidophilus	6.8 ± 0.3	7.0 ± 0.3

[a] Values are expressed as the mean ± standard deviation of two trials (duplicate samples were analysed in each trial).

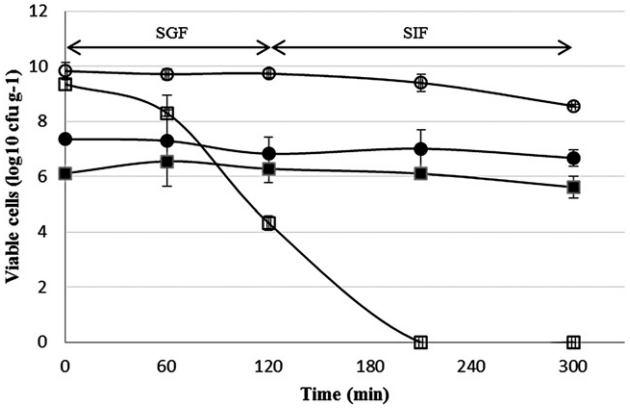

Fig. 3. Survival of free cells (○, *B. lactis*; □, *L. acidophilus*) and encapsulated cells (●, *B. lactis*; ■, *L. acidophilus*) after their exposure to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

and high concentrations of bile salts in the intestine (Sabikhi, Babu, Thompkinson, & Kapila, 2010). In the present study, the free and encapsulated cells of *B. lactis* were resistant to the SGF and SIF (Fig. 3); 87.1% of *B. lactis* cells remained viable with a 1.3 logarithmic cycle reduction in the population during 5 h of incubation when in its free form. When it was encapsulated, 90.7% of the cells remained viable with only a 0.7 logarithmic cycle reduction in the population. The microencapsulation by spray-chilling and the use of fat as the carrier, however, provided protection for *L. acidophilus* against simulated gastric and intestinal fluids. The free cells of *L. acidophilus* reached the method detection limit (10^2 cfu g^{-1}) in 210 min. When it was encapsulated, 91.8% of the cells remained viable during 5 h of incubation. The microencapsulation in question might provide even better protection to the cells when the microparticles were applied into a food matrix, considering that the presence of glucose enhanced the survival of probiotic lactobacilli during the gastric transit, as reported by Corcoran, Stanton, Fitzgerald, and Ross (2005). The results suggest that the lipid microparticles arrive in the intestine, where they are digested by lipases and the bile salts, and the probiotic cells have a high viability; therefore, the microorganisms are released in the vicinity of the site of action.

Other studies confirm the increase of the resistance of the microencapsulated probiotics when they are exposed to simulated gastric and intestinal fluids when compared with the survival of free cells. *L. acidophilus* that were encapsulated in sodium alginate were more resistant than the free cells when they were submitted to in vitro tests that simulated gastric and intestinal conditions (Kim et al., 2008; Mokarram, Mortazavi, Habibi Najafi, & Shahidi, 2009; Sabikhi et al., 2010). However, *L. acidophilus* that were encapsulated in calcium alginate (Favaro-Trindade & Grosso, 2000) or in a mixture of alginate and starch (Sultana et al., 2006) were not more resistant than the free cells when they were submitted to in vitro tests that simulated the stomach acidity and bile conditions.

In the present study, free *B. lactis* had higher gastrointestinal survival capacity than free *L. acidophilus*. A large variation in *Bifidobacterium* and *Lactobacillus* ability to resist acid and bile has been reported (Chung, Kim, Chun, & Ji, 1999; Clark, Cotton, & Martin, 1993; Köll et al., 2008). These properties are largely strain- and species-specific (Gomes & Malcata, 1999; Köll et al., 2008).

3.4. *Stability of the microencapsulated microorganisms during storage*

Cell damage and a loss of activity may occur during the processing and storage of the cells. Therefore, a suitable

Table 2
Stability of free and encapsulated *B. lactis* and *L. acidophilus* during 90 d of storage.[a]

Microorganism	Time (d)	Free (log_{10} cfu g^{-1})[b]			Encapsulated (log_{10} cfu g^{-1})		
		37 °C	7 °C	−18 °C	37 °C	7 °C	−18 °C
B. lactis	0	9.89 ± 0.16	9.89 ± 0.16	9.89 ± 0.16	7.36 ± 0.11	7.36 ± 0.11	7.36 ± 0.11
	30	7.35 ± 0.14	10.73 ± 0.36	9.65 ± 0.29	5.89 ± 0.16	8.08 ± 0.35	5.17 ± 0.18
	60	6.06 ± 0.36	6.13 ± 0.16	5.06 ± 0.05	5.30 ± 0.26	6.31 ± 0.01	7.59 ± 0.02
	90	2.09 ± 0.19	2.42 ± 0.06	5.36 ± 0.26	2.53 ± 0.12	2.54 ± 0.26	5.51 ± 0.36
L. acidophilus	0	9.53 ± 0.06	9.53 ± 0.06	9.53 ± 0.06	8.16 ± 0.11	8.16 ± 0.11	8.16 ± 0.11
	30	NS	4.98 ± 0.13	8.41 ± 0.09	6.99 ± 0.13	9.08 ± 0.16	8.00 ± 0.21
	60	NS	NS	2.00 ± 0.00	4.77 ± 0.10	4.35 ± 0.10	4.53 ± 0.02
	90	NS	NS	3.45 ± 0.15	5.11 ± 0.03	4.25 ± 0.26	6.56 ± 0.16

[a] Values are expressed as the mean ± standard deviation of two trials (duplicate samples were analysed in each trial).
[b] NS, no surviving cells.

microencapsulation process should ensure that the microorganism survives processing and that it remains viable during storage (Oliveira et al., 2007a, 2007b).

In the present study, according to results presented in Table 2, the free *B. lactis* population was unstable during storage; it displayed a logarithmic cycle reduction of 7.80 (37 °C), 7.47 (7 °C) and 4.53 log cfu g^{-1} (−18 °C), after 90 d of storage. Inactivation during storage can be related to a series of factors, such as the formation of free radicals in the presence of oxygen, fatty acid oxidation, and DNA damage (Castro, Teixeira, & Kirby, 1997). Microencapsulation improved the stability of *B. lactis* and presented a logarithmic cycle reduction of 4.83 (37 °C), 4.82 (7 °C) and 1.85 log cfu g^{-1} (−18 °C), during the same storage period. However, considering the minimum level of 10^6 cfu g^{-1} in the product (Talwalkar, Miller, Kailasapathy, & Nguyen, 2004), the microencapsulated cells that were stored at 37 °C had a shelf life of less than 30 d, and the shelf life was between 30 and 60 d when the cells were stored at 7 °C or −18 °C. These results suggest that cell stability increases with a decrease in temperature, and low temperatures can prevent crystal rearrangements and active ingredient exposure, which promotes a longer shelf life of the microparticle. Because the microorganisms are metabolically active in the microparticles at 7 °C and 37 °C, other explanations of their reduced viability are the production of compounds, such as metabolic acids and bacteriocins, and/or the absence of substrates, that may cause the inactivation of the viable cells in the microparticles. In addition, there are instances where viable counts during storage of free and encapsulated cells appear to increase due to chain breakup, but the reason for the apparent increases in cfu under our experimental conditions have not been ascertained.

Lipid microparticles also improved the stability of *L. acidophilus* during storage. The results were primarily observed at 37 °C and 7 °C; at these temperatures, the free cells lost their viability prior to 30 and 60 d of storage, respectively (Table 2). The microencapsulated cells had a shelf life between 30 and 60 d of storage at the same temperatures. A study by Oliveira et al. (2007b) showed that *L. acidophilus* displayed a greater viability at a storage temperature of 7 °C; however, in this work, the microcapsules were dehydrated. The probiotics that were encapsulated by complex coacervation and dehydrated by spouted bed showed viability when they were stored at 7 °C for 120 d, but they showed a loss in viability prior to 90 d of storage at 37 °C. In the present study, promising results were observed with freezing conditions, which assured 90 d of shelf life; in these conditions, microencapsulation increased the *L. acidophilus* viability from 36.6% (free cells) to 80.5% (microencapsulated cells). These results allow us to conclude that only freezing conditions provided a long and satisfactory microparticle shelf life. This result was likely observed because, in these conditions, the cells are in latent state. According to Rodrigues et al. (2011), high relative humidity, high temperature and longer storage periods were deleterious to the survival of probiotics. This result limits the application of these microparticles in food products.

4. Conclusions

The present study showed that spray-chilling technology, using a special vegetable fat as the carrier, produced solid lipid microparticles that were efficient in protecting the probiotics against the passage through gastric and intestinal fluids, and they could also be stored at low temperatures. In addition, the morphologies and sizes of the microparticles may facilitate the flow of material, while causing no harmful effects towards the food texture. Therefore, this method produces microparticles that seem to be a relatively innovative matrix for the application of probiotics with low cost and the potential of scale-up. A future challenge in this study is to improve the viability of the cells at refrigerated and room storage temperatures. Probably the use of protective solutions (carbohydrates, glycerol and pH adjustment) could help.

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