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Co- encapsulation of *Lactobacillus acidophilus* with inulin or polydextrose in solid lipid microparticles provides protection and improves stability

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ABSTRACT

The aims of this study were to produce and evaluate solid lipid microparticles (SLM) in which *Lactobacillus acidophilus* (La), a probiotic, was co-encapsulated with a prebiotics, either inulin (Inu) or polydextrose (Poly) using spray chilling technology. Morphological, chemical, and thermal characterisation of SLMs were conducted, along with survival assays to evaluate the resistance of the probiotic to the microencapsulation process, its resistance to exposure to simulated gastric fluids (SGF) and simulated intestinal fluids (SIF), and its stability throughout storage for 120 days at −18, 7 and 22 °C in a vacuum or with controlled relative humidity. Cell viability was not affected by the spray-chilling process. All of the microcapsules produced in the present study increased the survival rate of La exposed to SGF and SIF compared to that of free probiotic cells. Promising results were obtained when these microcapsules were stored refrigerated and frozen with a controlled relative humidity. This study indicated that combined spray chilling process, combined with the addition of a prebiotic component, specifically polydextrose is an interesting technology for the protection, delivery and improve stability of probiotics, which increases the potential of symbiotic SLMs. Scaling up the spray chilling technique will allow efficient encapsulation of probiotics in a lipid matrix.

1. Introduction

Probiotics are endowed with the ability to modulate of the intestinal microbiota, and the presence of prebiotics, ingredients that are selectively fermentable, exert a beneficial effect on the growth and/or activity of bacteria in the colon (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004; McCartney & Gibson, 2006; Roberfroid, 2007; Wells, Saulnier, & Gibson, 2008). There is a synergistic relationship between probiotics and prebiotics in which the prebiotics are consumed by probiotics as sources of carbon and energy, favouring their colonisation of the intestinal tract over colonisation by pathogenic microorganisms (Vernazza, Rabiu, & Gibson, 2006; Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008).

Microencapsulation has emerged as an alternative for protection of probiotics, providing a particular and convenient micro-environment for the encapsulated microorganism, enhancing their viability (Anal & Singh, 2007; Favaro-Trindade & Grosso, 2000; Rodrigues et al., 2011; Shah & Ravula, 2000; Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011), and enabling controlled release of cells in the intestinal tract (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; Favaro-Trindade, Heinemann, & Pedroso, 2011; Mandal, Puniya, & Singh, 2006; Oliveira et al., 2007a; Shoji et al., 2013). The choices of the method and materials are essential for an effective probiotic encapsulation strategy, and the use of gentle techniques, such as cold-induced gelation described by Nag, Han, and Singh (2011), as well as the appropriate materials, such as gastro-resistant polymers, biopolymers, and stearic acid (hydrophilic retardants), among others, are essential for efficient microencapsulation (Kanmani et al., 2011; Pimentel-González, Camposmontiel, Lobato-Calleros, Pedroza-Islas, & Vernoncarte, 2009).

Spray chilling is a microencapsulation technique that is based on the addition of the bioactive component to the molten carrier via dissolution, emulsion or dispersion. The mixture is passed through an atomizer nozzle, and when the nebulised material contacts an atmosphere refrigerated below the melting point of the matrix material (cold air chamber or liquid nitrogen), heat exchange occurs and the vehicle solidifies, creating solid lipid microparticles (SLMs) (Ilić et al., 2009).

Packaging probiotic microorganisms within solid lipid microparticles may protect them and is an interesting alternative to other conveyance systems, such as polymers and polysaccharides, because the microparticles will deliver the bioactive compound at approximately the melting point of the carrier material. SLMs produced with lipid materials are easily digested by the lipases in the intestines, releasing the probiotic in the vicinity of the intestines where they are required. The viability of microorganisms within lipid matrices was

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reported to be greater than that of free microorganisms (Lahtinen, Ouwehand, Salminen, Forssell, & Myllärinen, 2007; Pedroso, Thomazini, Heinemann, & Favaro-Trindade, 2012; Picot & Lacroix, 2003).

Encapsulation with lipid matrices is promising because lipids are likely to stabilise probiotics. Mandal, Hati, Puniya, Singh, and Singh (2012) found that number of viable free *Lactobacillus casei* NCDC 298 and *Lactobacillus casei* NCDC 298 encapsulated in a milk chocolate matrix did not differ during up to 60 days of storage under refrigerated conditions. They attributed this result to the high total solids in this matrix, including the fats and suggested that the fats had the ability to protect the probiotic cells. Hou, Lin, Wang, and Tzen (2003) encapsulated *Lactobacillus delbrueckii* ssp. in an artificial emulsion of sesame oil and observed that encapsulated bacteria performed better under simulated gastrointestinal conditions than did free cells. They concluded that the emulsion was an effective biocapsule for dairy products and Lahtinen et al. (2007) suggested that lipid matrix may protect the cells by blocking H^+ ions.

Pedroso et al. (2012) produced SLMs containing *B. lactis and L. acidophilus* using the spray chilling method. While analyzing the SLMs, they observed that encapsulated *L. acidophilus* cells were resistant to simulated gastric and intestinal fluids and that they had a shelf life of 30 and 60 days of storage at 37 °C and 7 °C, respectively. Therefore, the aims of this study were to produce solid lipid microparticles containing *L. acidophilus* and a prebiotic compound, to evaluate the ability of inulin and polydextrose to increase the viability of the probiotic throughout 120 days of storage at different temperatures, and to investigate probiotic survival in simulated gastric and intestinal fluids.

2. Materials and methods

2.1. Materials

A lipid carrier consisting of the fats obtained upon interesterification of fully hydrogenated palm and palm-kernel oil (GPPI) was used. The fats were kindly provided by Vigor (São Caetano do Sul, Brazil), their melting point is 43.34 °C.

A culture of *Lactobacillus acidophilus* (LAC-04) kindly provided by Danisco (Cotia, Brazil) was used as the active material. The prebiotics co-incorporated in the microparticles were inulin (Raftiline® ST, Beneo Orafti, Tienen, Belgium) and polydextrose (Litesse®, Danisco, Cotia, São Paulo, Brazil), which were kindly provided by Clariant (Suzano, Brazil) and Danisco (Cotia, Brazil), respectively.

2.2. Methods

2.2.1. Preparation of bacterial cell inoculum

The *L. acidophilus* culture was activated in sterilised MRS broth at 37 °C for 18 h. An aliquot of this culture was transferred to MRS broth and incubated for other 18 h at 37 °C in jars using an anaerobiosis system (Anaerobac, Probac, São Paulo-Brazil). This culture was centrifuged at 2400 *g* for 9 min at 4 ± 3 °C. The washed cells were resuspended in 2% sodium citrate at a concentration of approximately 10^9–10^{10} CFU/mL.

2.2.2. L. acidophilus cell counts

The number of viable La cells was determined using the pour plate technique, with MRS agar plates (DeMan Rogosa and Sharp) obtained from Acumedia (Indaiatuba, São Paulo, Brazil), according to the method described by Grosso and Favaro-Trindade (2004), with the modification suggested by Pedroso et al. (2012). One gram of SLMs was placed in 9 mL of 2% sodium citrate warmed at 52 ± 1 °C to completely dissolve the lipid matrix. The released cells were serially diluted in tubes containing warm 2% sodium citrate. Microaerophilic conditions were produced in anaerobiosis jars using the anaerobiosis generator systems. The jars were incubated at 37 °C ± 1°C for 72 h.

2.2.3. Preparation of solid lipid microparticles (SLM)

SLMs were produced according to the method described by Chambi, Alvim, Barrera-Arellano, and Grosso (2008), with some modifications. The inoculum (4%) was mixed with the prebiotic (3%), and the molten carrier was added. Suspensions of the mixtures (probiotic + prebiotic + lipid matrix) or (probiotic + lipid matrix) were produced using an Ultra-Turrax IKA® T-25 homogeniser (IKA, Staufen, Germany) at 7000 rpm for 60 s. The suspensions were atomised using a spray chiller (Labmaq, Ribeirão Preto, Brazil) equipped with a double fluid atomiser (Ø = 1.2 mm) and a cold chamber at 15 ± 2 °C (environmental variables: temperature and humidity), at a pressure of 5 bar.

Three formulations were produced: F1 (La without prebiotic), F2 (La + Inu), and F3 (La + Poly).

2.2.4. SLM characterisation

2.2.4.1. Microscopy. The morphologies of the SLMs were analysed using scanning electron microscopy (SEM). The SLMs were placed on pieces of double-faced carbon tape (Ted Pella, Inc., Redding-USA) that were fixed on aluminum stubs. Images were captured at a voltage of 5 kV and current of 1.750 mA. To analyse the internal morphologies of SLMs, they were frozen with liquid N_2 and sectioned. For confocal microscopy, the samples were examined using a Zeiss LSM 780-NLO confocal system with an Axio Observer Z.1 microscope (Carl Zeiss AG, Germany) and a 63x/1.4 NA oil plan apochromatic DIC objective lens. Images in a 1024 × 1024 format were captured using 605 nm laser lines for excitation, with pinholes set to 1 airy unit for each channel. The bacteria were stained with SYTO 9, a component of the LIVE/DEADs BacLight bacterial viability kit (Molecular Probes, Eugene, Oregon USA).

2.2.4.2. Particle size. A Shimadzu Sald-201V particle size analyser (Kyoto, Japan) that employs laser diffraction was used to determine the sizes and size distributions of the SLMs. The SLMs were dispersed in ethyl alcohol (Synth, Brazil) and stabilised for 5 min before the assay.

2.2.4.3. Differential scanning calorimetry (DSC). Differential scanning calorimetry was conducted using a DSC M2010 system (TA Instruments, Newcastle, USA). Approximately 10 mg of each sample (GPPI/SLM) was placed in an aluminum capsule and gradually heated (10 °C/min) to temperatures between −50 and 100 °C in an inert atmosphere (45 mL/min of N_2). An empty capsule was used as the reference. The data were analysed using Universal Analysis 2000 version 3.9[a] (TA Instruments) (Favaro-Trindade, Santana, Monterrey-Quintero, Trindade, & Netto, 2010).

2.2.4.4. Fourier transform infrared (FTIR) spectroscopy. The pure ingredients and the SLMs were characterised by FTIR spectroscopy in the 4000 to 600 cm^{-1} region using a Perkin Elmer FTIR spectrometer (Massachusetts/USA) and Spectrum One version 5.3.1 software. The probiotic sample was suspended in 2% sodium citrate.

2.2.4.5. X-ray powder diffraction. The individual components and microparticles were analysed using the X-ray powder diffraction technique. An AXS Analytical X-Ray Systems Siemens D 5005 (Germany) diffractometer with Cu K alpha radiation (λ = 1.54056A°) was used with the voltage was 40 kV and current set at 40 mA. The scanning angle ranged from 3 to 90° of 2θ, 2°/min and 0.033°/s.

2.2.5. Viability of encapsulated microorganisms during storage

For the stability studies, the microparticles were stored at three different temperatures (−18, 7, and 22 °C) in a vacuum or with the relative humidity controlled with lithium chloride (LiCl), as described by Heidebach, Forst, and Kulozik (2010). The SLMs were stored in

hermetically sealed glass flasks or in open flasks in hermetically sealed containers containing LiCl, and later on stored in a BOD that was set at the three temperatures. The number of viable cells was counted after 7, 30, 60, 90, and 120 days of storage.

2.2.6. In vitro evaluation of gastric and intestinal fluids

The *in vitro* resistance of free and microencapsulated microorganisms to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was evaluated using the methods described by Gbassi, Vandamme, Ennahar, and Marchioni (2009). The SGF was composed of 9 g/L of sodium chloride (Synth, Diadema, Brazil) and 3 g/L of porcine stomach pepsin (Sigma-Aldrich, St. Louis, MO, USA) in distilled water and was adjusted with HCl to pH 1.8. The SIF was prepared with 9 g/L of sodium chloride (Synth, Diadema, Brazil), 10 g/L of pancreatin, the same amount of bovine pancreas trypsin (Sigma-Aldrich, St. Louis, MO, USA), and 3 g/L of bile salts (Oxgall, Difco, Hampshire, UK) suspended in distilled water. The pH was adjusted to 6.5 with sodium hydroxide (Synth, Diadema, Brazil). The microorganisms were counted after 0, 60, and 120 min in SGF, and after 0, 90, and 180 min in SIF.

2.2.7. Statistical analysis

A completely randomised design (CRD) using a $3 \times 2 \times 3 \times 6$ factorial treatment combination was adopted to evaluate the variable-dependent numbers of viable *L. acidophilus* cells determined in the stability assays according to the prebiotic (without prebiotic or with inulin or polydextrose), the type of storage (vacuum or relative humidity), the temperature (−18, 7 or 22 °C), and the length of storage (0, 7, 30, 60, 90 or 120 days). Because this statistical model showed significant effects for the quadruple interactions, developments were assessed using Tukey's test within each prebiotic/type of storage/temperature combination, prebiotic/temperature/length of storage, type of storage/temperature/length of storage and prebiotic/type of storage/length of storage combination. All of the tests were performed using Statistical Analysis System© software (SAS, 2005), and the PROC MIXED procedure.

3. Results and discussion

3.1. SLM characterisation

The images captured using scanning electron microscopy, such as Fig. 1, demonstrated the spherical shape of the SLMs obtained by spray chilling, and showed that they have a relatively smooth continuous surface, without pronounced cracks or pores. Additionally, because solvent did not have to be evaporated as in the spray drying technique, the structures were not hollow, but completely packed, as shown in Fig. 1(A).

The images obtained using confocal microscopy, such as Fig. 1(B), showed the bacteria immobilised in the lipid carrier, appearing either green or red. The live microorganisms are green and dead ones are red. Moreover, these bacilli displayed their characteristic shape. The main inconvenience of these microparticles is that microorganisms appear to be dispersed throughout them and may be at the surface, where they should be susceptible to the adverse conditions of the environment. The inclusion of inulin or polydextrose did not affect the morphology of the SLMs; this observation is in agreement with that of Fritzen-Freire et al. (2012), who microencapsulated bifidobacteria in the presence of prebiotics-inulin, oligofructose, and oligofructose-enriched inulin- using spray drying technology.

3.2. Particle size

One peculiarity of the encapsulation of probiotic microorganisms is their relatively large size, typically 1–4 μm, which limits the encapsulation for relatively small particles (Anal & Singh, 2007). Moreover, very large particles may negatively affect organoleptic characteristics (Hansen, Allan-Wojtas, Jin, & Paulson, 2002). However, Chandramoulia, Kailasapathya, Peirisb, and Jonesb (2004) observed that the greater the diameter of the capsule, the greater the viability of *Lactobacillus* spp., in simulated gastric conditions. Table 1 presents mean diameters and diameters corresponding to the accumulated distribution of 10, 50 and 90%, which represents the average particle diameter considering that 10% of total particles, 50% of total particles, and 90% of total of the particles. The span value $((d(0.9) - d(0.1))/d(0.5))$ is a measurement of the width of size distribution (Ilić et al., 2009). The values for the three types of microparticles were not significantly different ($p < 0.05$). Therefore, it is possible to conclude that the prebiotics used in the production of these microparticles did not affect their size. According to the span values, the width of microparticle distribution was not changed by the presence of inulin or polydextrose.

3.3. DSC

Table 2 shows the onset and maximum temperatures, as well as the enthalpy for the fat, microorganism inoculum, and the SLMs.

The enthalpy values for the lipid carrier and the three types of SLMs were relatively similar, 123.4 J/g and 118.9; 111.0 and 123.0 J/g, respectively. Silva et al. (2011) studied solid–lipid nanoparticles charged with risperidone and found that the enthalpy values were lower for all of the formulations compared to that of the encapsulating agent (Imwitor®

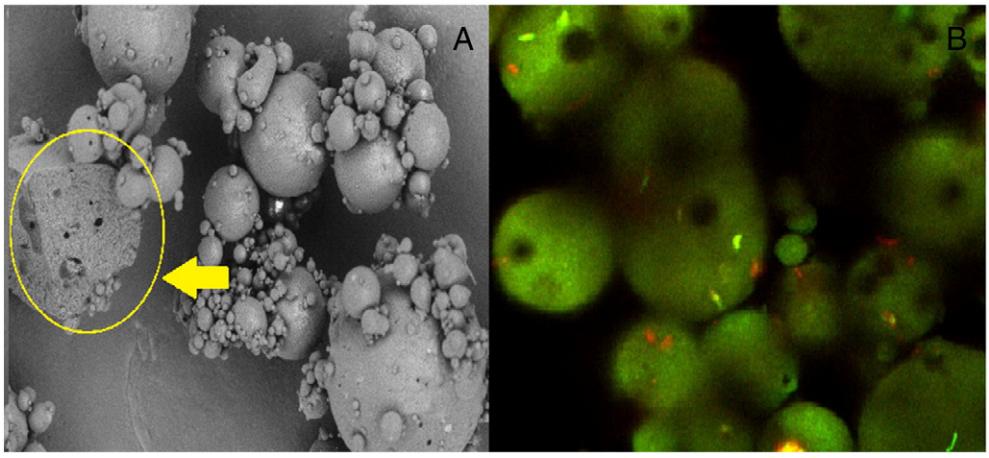

Fig. 1. Scanning electron microscopy image (A) of the microparticles containing *L. acidophilus* (500× magnification) and confocal microscopy image (B) of SLMs containing *L. acidophilus* that were prepared by spray-chilling; the bacteria are stained with SYTO9 (500× magnification). The arrow points to internal morphology of SLM, matrix type.

Table 1
Effect of different formulations on the particle size distribution of the microparticles (average ± SD; n = 3).

Formulation	D(4,3)* (μm)	D(0,1)** (μm)	D(0,5)*** (μm)	D(0,9)**** (μm)	SPAN
F1	65.2 ± 8.1[a]	38.2 ± 11.6	70.0 ± 3.7	99.7 ± 0.4	0.879 ± 0.198
F2	63.7 ± 5.1[a]	37.4 ± 5.6	68.9 ± 2.5	99.9 ± 1.3	0.886 ± 0.099
F3	66.1 ± 3.9[a]	41.8 ± 6.3	70.0 ± 1.7	98.0 ± 3.9	0.848 ± 0.148

F1: *L. acidophilus*; F2: *L. acidophilus* + inulin; F3: *L. acidophilus* + polydextrose.

900K). However, in the study of Silva et al. (2011), nanocapsules were produced by first adding surfactants to obtain pre-emulsions and then using two different techniques ultrasound (US) and high-pressure homogenisation (HPH). The melting temperatures of the formulations were very similar to each other and slightly higher than the melting temperature of pure fat, which is 43.7 °C. In fact, the melting temperatures for the formulations were expected to be similar because the lipid matrix used was the same for all of the formulations. Neither the inoculum, or the inoculum and the prebiotic, interact with the lipid matrix of the SLMs and, therefore, they did not change the polymorphic behavior of the mixtures used to produce them.

As for the possibility of melting, prematurely, the melting temperatures observed for the formulations were between 47.27 and 47.58 °C, which would make them physically stable at room temperature and in the mouth.

3.4. X-ray powder diffraction

Additional information on the solid state structures of SLMs was obtained using X-ray powder diffraction. Fig. 2 shows the diffraction profiles of the three formulations tested, when they were freshly prepared and after 90 days of storage. Because with the SLM have a lipid matrix, a trend for polymorphic reorganisation should be considered for more energetically favourable levels, and this behavior may lead to the expulsion of the bioactive ingredient when it attains a more crystalline arrangement (Gamboa, Gonçalves, & Grosso, 2011; Jenning, Thünemann, & Gohla, 2000; Müller, Radtke, & Wissing, 2002a, 2002b; Schubert & Müller-Goymann, 2005). Fig. 2 shows that the refractograms had similar shapes, independently of the formulations or the period of storage. The diffraction patterns of the SLMs are very similar to each other, indicating that no polymorphic changes occurred during storage. The behavior of lipid material observed in this study was similar to that reported in the literature, which is generally associated with a polymorphic β form that is characteristic of triacylglycerol and fatty acids (Gamboa et al., 2011).

3.5. FTIR

The FTIR assay detected possible interactions between the encapsulated materials and the carrier. Fig. 3 shows peaks of interesterified palm and palm-kernel oil at 1738, 2851, and 2919 cm^{-1} in the5(B) spectrum. These peaks are associated with the presence of carbonylated

Table 2
DSC parameters for the encapsulating matrix, bioactive material (inoculum) and formulations prepared with *L. acidophilus* with or without addition of a prebiotic.

Sample	Peak (°C)	OnSet (°C)	Enthalpy (J/g)
Fat	43.34	32.28	123.4
Inoculum	47.82	47.24	2.89
F1	47.43	39.29	118.9
F2	47.27	34.98	111.0
F3	47.58	34.52	123.0

F1: *L. acidophilus*; F2: *L. acidophilus* + inulin; F3: *L. acidophilus* + polydextrose.

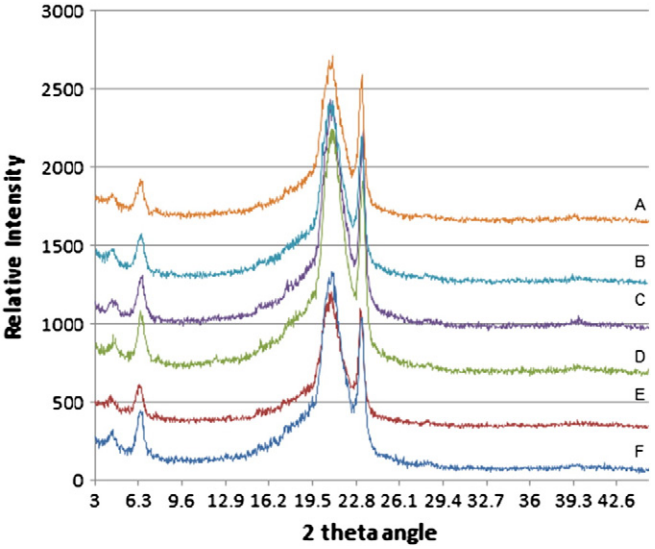

Fig. 2. X-ray diffraction patterns of F1-0 day (A), F1-90 days (B), F2-0 day (C), F2-90 days (D), F3-0 day (E) and F3-90 days (F).

compounds, more precisely, the vibration of COOH groups, and the peak at 1738 cm^{-1} is related to C═O vibration. Passerini, Albertini, Perissutti, and Rodriguez (2006) used the lipid matrix Gelucire 50/13 to encapsulate a drug (Praziquantel), and observed a large band in the 3650–3100 cm^{-1} region that was related to the vibration of the O–H bonds in the COOH groups.

The infrared spectrum of inulin 3 (C-solid line) demonstrated the polysaccharide nature of the C–O–C bond that is characteristic of carbohydrates between 1200 and 900 cm^{-1}, which confirmed that the monomers had bound to one another to form polymers. The band for the O–H bond in the monosaccharide structure is observed between 3600 and 3000 cm^{-1}. An angular deformation of the bond at 1639 cm^{-1} is also observed. Asymmetric stretching of the CH_2 group is observed between 2900 and 2950 cm^{-1} (Silverstein, Webster, & Kiemle, 2006).

The spectrum for polydextrose 3(C-broken line) displays a pronounced vibration related to C–O–C glycosidic bond (1202–927 cm^{-1}), C═O stretching vibration of aldehyde (1659 cm^{-1}), O–H stretching vibrations (3640–2978 cm^{-1}) and a C–H stretching vibration at 2946 cm^{-1} (Mickova, Copikova, & Synytsya, 2007).

Analysis of the infrared spectra of the SLMs, shown in Fig. 3(D,E,F), revealed that none of the peaks characteristic of the encapsulated material (prebiotic and probiotic) or the carrier were changed, suggesting the absence of significant interactions.

3.6. Resistance to encapsulation by spray chilling

Table 3 shows the values pertaining of resistance to the production process in all the formulations. Based on these results, the microorganisms are highly resistant to the spray chilling process, that is, atomisation and cooling of the molten mixture to maintain the viability of the *L. acidophilus* cells, both in the mixture with molten carrier and in the SLMs. It is conceivable that spray chilling had very little effect on cellular integrity. A similar behavior was reported by Pedroso et al. (2012), who employed the same encapsulation process. Ultraturrax homogenisation, as well as the exposure to heat when the cells were added to the molten fat, did not seem to have had strong impact on cell survival, considering the initial count of the microorganisms in the inoculum and their dilution at 4 g/100 mL in the molten mixture.

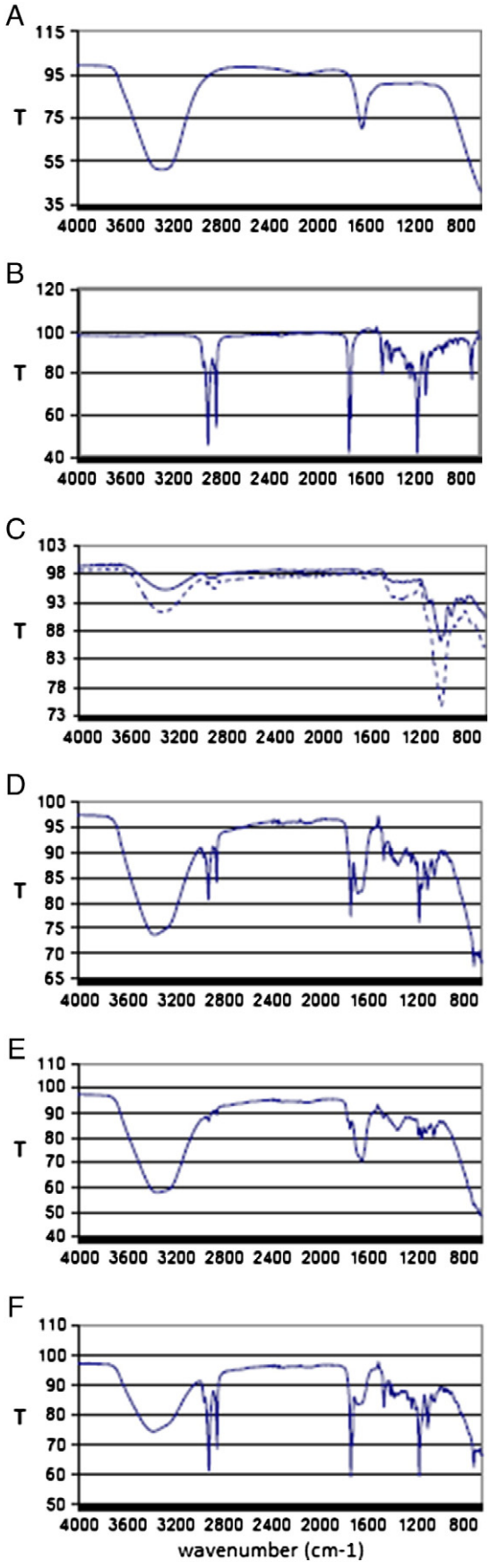

Fig. 3. FT-IR spectra of *L. acidophilus* (A), palm oil and palm-kernel oil (GPPl) (B), Inulin (solid line) and polydextrose (broken line) (C), F1(D), F2(E) and F3 (F).

Table 3
Resistance of *L. acidophilus* to the spray-chilling process.

Formulation	Viable cells (count in $\log_{10}$ cfu g^{-1})		
	Inoculum	Suspension [a]	SLMs[b]
F1	10.46 ± 0.30	8.67 ± 0.31	8.34 ± 0.38
F2		8.53 ± 0.34	8.29 ± 0.27
F3		8.57 ± 0.26	8.32 ± 0.09

F1: *L. acidophilus*; F2: *L. acidophilus* + inulin; F3: *L. acidophilus* + polydextrose.
[a] Before atomisation.
[b] After the spray-chilling process.

3.7. Resistance to gastrointestinal fluids

In the present study, microencapsulation protected La from simulated gastrointestinal conditions (Fig. 4). The free cells count reached the limit of the method (10^{-2} CFU/g) by 210 min in the assay, demonstrating that the cells were susceptible to the simulated conditions. Although free viable cells were not detectable after 210 min, approximately 60% of the La cells in the SLMs produced with or without a prebiotic were found to be viable. There was a reduction of 2.99; 2.79; 2.84 log cycles for formulations F1, F2, and F3, respectively, by 300 min of treatment.

Light microscopy (Fig. 4) showed that the SLMs were intact, well-defined spheres at the start of the simulated gastrointestinal conditions assays. The most significant disintegration of SLMs occurred when they were exposed to simulated intestinal fluid (SIF), where the presence of pancreatin, trypsin, and bile salts, together with increased pH (6.5), created conditions favourable for the rupture/disintegration of SLMs, due to their lipid content.

The release of the active components (bacteria and prebiotics) was correlated with a more drastic reduction in the viable cell count because probiotics that are not protected in the SLMs are more susceptible to external conditions.

SGF-treatment did not significantly reduce ($p \leq 0.05$) the viable cell count associated with the SLMs containing polydextrose (60 to 120 min) or the SLMs lacking a prebiotic (0 to 60 min), but a significant reduction in cell viability was observed for all of the SLMs that passed from SGF to SIF and for those incubated in intestinal fluid (120 to 210 min and 210 to 300 min). At the end of the experiment, the cell counts for the F2 and F3 formulations and for the F1 and F2 formulations were not significantly different, whereas the cells in the F2 and F3 formulations demonstrated better resistance to the fluids than did those in the F1 formulation. These results showed that the F2 and F3 formulations could be used to improve the resistance of La to the simulated gastrointestinal conditions.

There was a reduction of 2.19, 1.75 and 1.73 log cycles for F1, F2, and F3, respectively. There was a significant reduction in viable cells counts when probiotics were exposed to bile salts. The antimicrobial nature of bile salts is related to its detergent property, which dissolves microorganism membranes, and its amphiphilic nature makes it strongly inhibitory for the gastrointestinal tract (Madureira, Amorim, Gomes, Pintado, & Malcata, 2011; Senaka Ranadheera, Evans, Adams, & Baines, 2012).

Kim et al. (2008) observed similar results. They reported a 3 log-cycle reduction in viable cells when microparticles of *L. acidophilus* ATCC 43121 microencapsulated with sodium alginate were exposed to artificial gastric juices at pH 1.2 and 1.5. Other studies also found better survival for encapsulated L. acidophilus than for free cells when they were exposed to simulated gastric and intestinal solutions (Favaro-Trindade & Grosso, 2002; Kim et al., 2008; Sabikhi, Babu, Thompkinson, & Kapila, 2010).

3.8. Viability

High relative humidity negatively affects the viability of microorganisms in capsules during storage because the increase in water

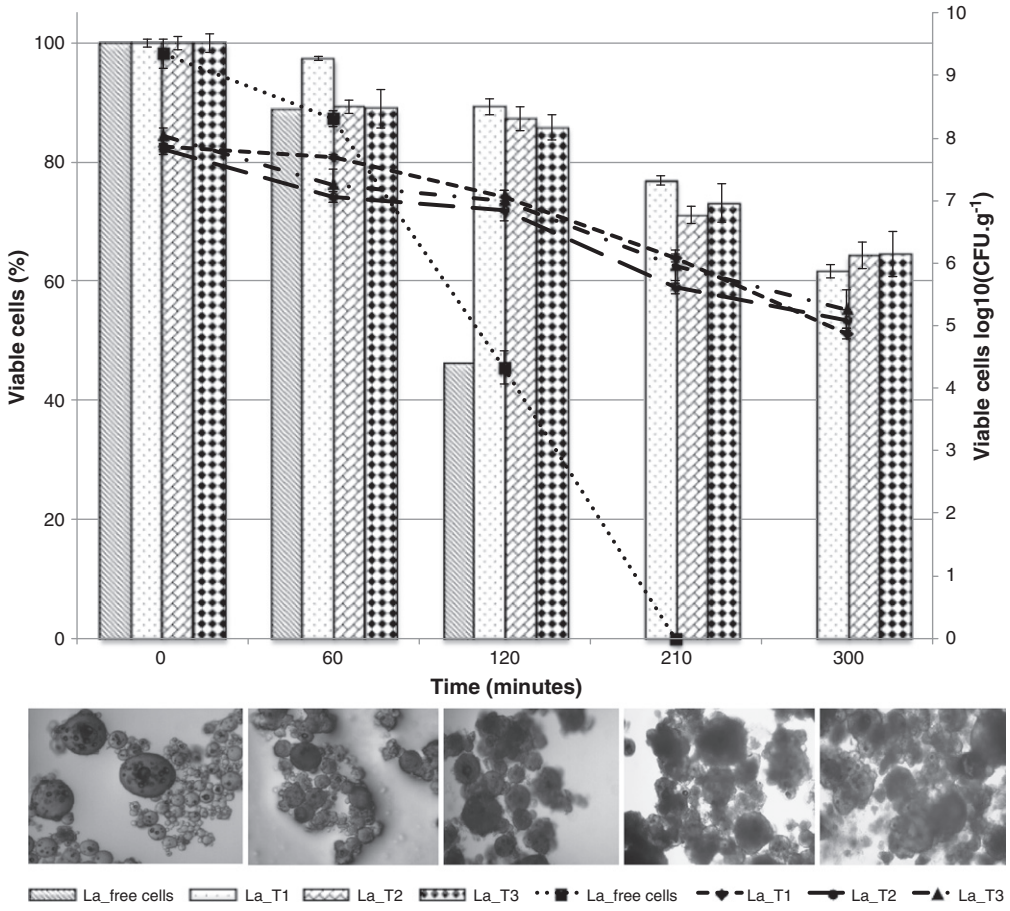

Fig. 4. Survival of free and microencapsulated *Lactobacillus acidophilus* cells after their exposure to simulated gastric fluid (0–120 min) and simulated intestinal fluid (120–300 min). The bars represent the survival of the probiotic as percentages and the lines represent the survival in Log (CFU/g).

content is detrimental to the microorganisms due to the acceleration of oxidative process (Rodrigues et al., 2011; Teixeira, Castro, Malcata, & Kirby, 1995).

Therefore, in this study, we attempted to decrease Aw of the microparticles by incorporating prebiotics to inhibit the metabolic activity of the immobilised probiotic microorganisms and thereby improve their stability during storage. A determinate number of viable probiotic cells must be consumed (10^6–10^7 CFU/g) for them to play a significant role when they eventually reach the intestine (Sabikhi et al., 2010; Talwalkar, Miller, Kailasapathy, & Nguyen, 2004).

Table 4
Effects of the storage parameters on the stability of microencapsulated *L. acidophilus* throughout 120 days of storage.

Preb	Storage conditions	T (°C)	Viable cells (log cfu g^{-1})											
			0 days	7 days	30 days	60 days	90 days	120 days
WP	UR (11%)	−18	8.381 ± 0.640 a A	7.513 ± 0.107 b A	7.028 ± 0.158 b A	5.639 ± 0.142 c A	5.271 ± 0.237 c A	4.024 ± 0.088 d A
WP	UR (11%)	7	8.381 ± 0.640 a A	7.155 ± 0.295 b A	5.021 ± 0.829 c B	4.529 ± 0.824 cd B	4.061 ± 0.851 d B	3.085 ± 0.176 e B
WP	UR (11%)	22	8.381 ± 0.640 a A	4.233 ± 0.487 b B	3.470 ± 0.723 c C	2.314 ± 0.160 d C	1.435 ± 0.298 e C	NS f C
WP	Vácuo	−18	8.381 ± 0.640 a A	7.519 ± 0.372 b A	6.725 ± 0.172 c A	5.830 ± 0.082 d A	5.081 ± 0.093 e A	3.757 ± 0.541 f A
WP	Vácuo	7	8.381 ± 0.640 a A	4.993 ± 0.090 b B	2.941 ± 0.650 c B	2.000 ± 0.990 d B	NS e B	NS e B
WP	Vácuo	22	8.381 ± 0.640 a A	3.709 ± 0.176 b C	2.319 ± 0.584 c B	1.724 ± 0.345 c B	NS d B	NS d B
Inu	UR (11%)	−18	8.194 ± 0.444 a A	7.815 ± 0.268 a A	6.751 ± 0.121 b A	6.432 ± 0.193 bc A	6.063 ± 0.211 cd A	5.708 ± 0.204 d A
Inu	UR (11%)	7	8.194 ± 0.444 a A	6.993 ± 0.090 b B	6.361 ± 0.175 c A	4.843 ± 0.146 d B	4.450 ± 0.161 de B	3.954 ± 0.041 e B
Inu	UR (11%)	22	8.194 ± 0.444 a A	6.249 ± 0.083 b C	5.079 ± 0.197 c B	4.554 ± 0.149 cd B	4.256 ± 0.091 d B	3.603 ± 0.512 e B
Inu	Vácuo	−18	8.194 ± 0.444 a A	7.746 ± 0.265 ab A	7.121 ± 0.059 b A	5.654 ± 0.097 c A	5.117 ± 0.199 c A	4.462 ± 0.153 d A
Inu	Vácuo	7	8.194 ± 0.444 a A	4.992 ± 0.450 b B	2.464 ± 1.415 c B	0.956 ± 1.105 d B	NS e B	NS e B
Inu	Vácuo	22	8.194 ± 0.444 a A	3.759 ± 0.153 b C	1.737 ± 0.744 c C	NS d C	NS d B	NS d B
Poly	UR (11%)	−18	8.964 ± 0.331 a A	8.472 ± 0.545 ab A	7.979 ± 0.393 bc A	7.618 ± 0.192 cd A	7.125 ± 0.063 d A	6.997 ± 0.085 d A
Poly	UR (11%)	7	8.964 ± 0.331 a A	8.314 ± 0.225 b A	7.491 ± 0.370 c AB	7.397 ± 0.199 cd A	7.068 ± 0.037 cd A	6.812 ± 0.039 d A
Poly	UR (11%)	22	8.964 ± 0.331 a A	8.111 ± 0.407 b A	6.915 ± 0.853 c B	6.009 ± 0.724 d B	5.554 ± 0.444 de B	4.939 ± 0.586 e B
Poly	Vácuo	−18	8.964 ± 0.331 a A	7.975 ± 0.273 b A	7.523 ± 0.118 bc A	6.895 ± 0.170 cd A	6.388 ± 0.250 d A	5.517 ± 0.400 e A
Poly	Vácuo	7	8.964 ± 0.331 a A	6.006 ± 0.963 b B	2.386 ± 1.370 c B	NS d B	NS d B	NS d B
Poly	Vácuo	22	8.964 ± 0.331 a A	4.330 ± 0.402 b C	2.007 ± 1.259 c B	NS d B	NS d B	NS d B

Viable cell counts for each prebiotic-storage condition- temperature combination were affected by duration of storage; values denoted with different lowercase letters within the rows are significantly different (Tukey test: $p \leq 0.05$). Viable cell counts for each prebiotic-storage condition–duration of storage combination were affected by the storage temperature; values denoted with different capital letters within the columns are significantly different (Tukey test: $p \leq 0.05$).
NS: no surviving cells.

Table 5
Effects of the storage parameters on the stability of microencapsulated *L. acidophilus* throughout 120 days of storage.

Preb	Storage conditions	T (°C)	Viable cells (log cfu g^{-1})																					
			0 days			7 days			30 days			60 days			90 days			120 days						
WP	UR (11%)	−18	8.381 ± 0.640	a	A	7.513 ± 0.107	b	A	7.028 ± 0.158	b	A	5.639 ± 0.142	c	A	5.271 ± 0.237	c	A	4.024 ± 0.088	c	A			
Inu	UR (11%)	−18	8.194 ± 0.444	b	A	7.815 ± 0.268	b	A	6.751 ± 0.121	b	A	6.432 ± 0.193	b	A	6.063 ± 0.211	b	A	5.708 ± 0.204	b	A			
Poly	UR (11%)	−18	8.964 ± 0.331	a	A	8.472 ± 0.545	a	A	7.979 ± 0.393	a	A	7.618 ± 0.192	a	A	7.125 ± 0.063	a	A	6.997 ± 0.085	a	A			
WP	UR (11%)	7	8.381 ± 0.640	ab	A	7.155 ± 0.295	b	A	5.021 ± 0.829	c	A	4.529 ± 0.824	b	A	4.061 ± 0.851	b	A	3.085 ± 0.176	c	A			
Inu	UR (11%)	7	8.194 ± 0.444	b	A	6.993 ± 0.090	b	A	6.361 ± 0.175	b	A	4.843 ± 0.146	b	A	4.450 ± 0.161	b	A	3.954 ± 0.041	b	A			
Poly	UR (11%)	7	8.964 ± 0.331	a	A	8.314 ± 0.225	a	A	7.491 ± 0.370	a	A	7.397 ± 0.199	a	A	7.068 ± 0.037	a	A	6.812 ± 0.039	a	A			
WP	UR (11%)	22	8.381 ± 0.640	ab	A	4.233 ± 0.487	c	A	3.470 ± 0.723	c	A	2.314 ± 0.160	c	A	1.435 ± 0.298	c	A	NS					
Inu	UR (11%)	22	8.194 ± 0.444	b	A	6.249 ± 0.083	b	A	5.079 ± 0.197	b	A	4.554 ± 0.149	b	A	4.256 ± 0.091	b	A	3.603 ± 0.512	b	A			
Poly	UR (11%)	22	8.964 ± 0.331	a	A	8.111 ± 0.407	a	A	6.915 ± 0.853	a	A	6.009 ± 0.724	a	A	5.554 ± 0.444	a	A	4.939 ± 0.586	a	A			
WP	Vácuo	−18	8.381 ± 0.640	ab	A	7.519 ± 0.372	a	A	6.725 ± 0.172	b	A	5.830 ± 0.082	b	A	5.081 ± 0.093	b	A	3.757 ± 0.541	c	A			
Inu	Vácuo	−18	8.194 ± 0.444	b	A	7.746 ± 0.265	a	A	7.121 ± 0.059	ab	A	5.654 ± 0.097	b	B	5.117 ± 0.199	b	B	4.462 ± 0.153	b	B			
Poly	Vácuo	−18	8.964 ± 0.331	a	A	7.975 ± 0.273	a	A	7.523 ± 0.118	a	A	6.895 ± 0.170	a	B	6.388 ± 0.250	a	B	5.517 ± 0.400	a	B			
WP	Vácuo	7	8.381 ± 0.640	ab	A	4.993 ± 0.090	b	B	2.941 ± 0.650	a	B	2.000 ± 0.990	a	B	NS			NS					
Inu	Vácuo	7	8.194 ± 0.444	b	A	4.992 ± 0.450	b	B	2.464 ± 1.415	a	B	0.956 ± 1.105	b	B	NS			NS					
Poly	Vácuo	7	8.964 ± 0.331	a	A	6.006 ± 0.963	a	B	2.386 ± 1.370	a	B	NS			NS			NS					
WP	Vácuo	22	8.381 ± 0.640	ab	A	3.709 ± 0.176	a	A	2.319 ± 0.584	a	B	1.724 ± 0.345	a	A	NS			NS					
Inu	Vácuo	22	8.194 ± 0.444	b	A	3.759 ± 0.153	a	B	1.737 ± 0.744	a	B	NS			NS			NS					
Poly	Vácuo	22	8.964 ± 0.331	a	A	4.330 ± 0.402	a	B	2.007 ± 1.259	a	B	NS			NS			NS					

Viable cell counts for each storage conditions–temperature-duration of storage combinations were affected by prebiotic; values denoted with different lowercase letters within the rows are significantly different (Tukey test: $p \leq 0.05$). Viable cell counts for each prebiotic-temperature-duration of storage combination were affected by the storage condition; values denoted with different capital letters within the columns are significantly different (Tukey test: $p \leq 0.05$).
NS: no surviving cells.

In the present study, according to the results presented in Tables 4 and 5, the F3 formulation stored in UR at −18 °C and 7 °C for 120 days would provide the number of viable cells required, and they presented a logarithmic cycle reduction of 1.967 and 2.152, respectively. The good results obtained for low temperature storage may be explained by the microorganisms being maintained in a latent state and to the low temperature preventing crystal rearrangements in the lipid matrix and thus expulsion of the bioactive component (Pedroso et al., 2012).

In a previous study, *L. acidophilus* retained greater viability at a storage temperature of 7 °C (120 days), as observed in this study for the F3 formulation, although in the previous study, the microcapsules were produced by complex coacervation and were dehydrated by the spouted bed method (Oliveira et al., 2007b).

The effects of adding inulin or polydextrose on the survival of La are presented in Table 5. The data show that the presence of polydextrose increased cell viability ($p \leq 0.05$) compared with the presence of inulin or no prebiotic during storage with controlled relative humidity at −18, 7 and 22 °C. The cell viability values for F1, F2 and F3 formulations stored under vacuum for 30 days at 7 or 22 °C were not significantly different, whereas the cell viability after frozen storage for 30 to 120 days was significantly greater for the F3 formulation.

For all the formulations tested, it was observed that storage at the lowest temperature studied led to the best cell viability, as shown in Table 4. There is a consensus that temperatures close to 0 °C improve the rates of cell viability because lower temperatures reduce the rates of chemical reactions that are detrimental to the microorganisms, such as fatty acid oxidation (Corcoran, Ross, Fitzgerald, & Stanton, 2004; Teixeira et al., 1995).

For F1, the storage at freezing temperature (−18 °C) showed the best rate of viable cells, with counts greater than 6 log cycles, both under vacuum and controlled relative humidity up to 30 days. However, for the other temperatures analysed, stability was inadequate, once there was a great reduction in the number of viable cells in 7 days, except for relative humidity at 7 °C, whose counts decreased in 30 days, this result is possibly related to greater metabolic activity of the microorganisms when stored at 7 and 37 °C. High temperatures can lead to production of metabolic acids and bacteriocins, and/or the loss of substrates, which would explain the inactivation of the viable cells in the microparticles during storage.

F2 showed the best performance at −18 °C, under controlled relative humidity, with satisfactory counts up to 90 days; up to 30 days when stored under controlled relative humidity at 7 °C; and under vacuum at −18 °C. Finally, F3 seemed to present the best responses in viability during storage in controlled relative humidity (11%), where counts over 10^6 were recorded up to 120 days at −18 °C and 7 °C, and up to 60 days at 22 °C, whereas under vacuum counts remained acceptable for 90 days at −18 °C.

It is known that not only temperature, but also relative humidity, is determinant for probiotic survival during storage. Moreover, the Aw values are balanced at room relative humidity. Thus, storage at high relative humidity increases the water concentration, which is detrimental to the survival of probiotics. On the other hand, at very low relative humidity, removal of water from the cells may increase the possibility of damage. Therefore, intermediate relative humidity levels, between 7 and 11%, were reported to be the ideal values for bacterial survival (Castro, Teixeira, & Kirby, 1995).

Pedroso et al. showed that microencapsulated *L. acidophilus* had a shelf life of 30 and 60 days at 7 and 37 °C, respectively. Although this study used the same encapsulation process and lipid matrix they used, they did not add prebiotic components or store the microcapsules with a controlled relative humidity, which explains the lower stability observed in their study. The presence of a prebiotic and the controlled storage conditions improved the stability of *L. acidophilus*.

4. Conclusions

Symbiotic solid lipid microparticles are potential vehicles of probiotic microorganisms and prebiotic compounds. The symbiotic SLM-based systems could protect to *L. acidophilus* cells from the effects of gastric and intestinal fluids and release them in the intestines during fat digestion.

SLMs improve the viability of *L. acidophilus* during storage at freezing or refrigeration temperatures with controlled relative humidity (11%). The best formulation studied combined *L. acidophilus* and polydextrose because this system maintained the viability of the stored microorganisms viable for 120 days.

The SLMs produced in this study are an interesting vehicle that can be applied by the food industry, given their specific morphology and insoluble nature. Issues remain to be investigated, such as the effects of different levels of prebiotics on the morphology of SLMs

and the probiotic cell viability as well as the effect of other lipid matrices that may prolong probiotic cell viability during storage, thereby favouring the application of SLMs in food products.

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