Viability of *Lactobacillus acidophilus* La5 in pectin–whey protein microparticles during exposure to simulated gastrointestinal conditions

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**Abstract**

The objective of this study was to evaluate whether the coating of pectin microparticles containing *Lactobacillus acidophilus* La5 with whey protein heat treated or without heat treatment affects the viability of probiotics when exposed to conditions simulating the passage through the gastrointestinal tract. The microparticles were produced by ionotropic gelation and coated with whey protein by electrostatic interaction. The microparticle morphology and the viability of *L. acidophilus* La5 during exposure to simulated gastric (pH 1.2 or 3.0) and intestinal (pH 7.0) conditions were evaluated. There was no significant difference in the viability of *L. acidophilus* La5 after encapsulation, which was in the order of 8 log CFU/g for all the microparticles. The pectin microparticles remained intact when exposed to simulated gastrointestinal conditions at pH 1.2, 3.0 and after 300 min at pH 7.0. On the other hand, both microparticles coated with whey protein heat treated or without heat treatment have remained intact for 120 min exposure to simulated gastric juice but have disintegrated after 300 min exposure to simulated intestinal juice (pH 7.0). This occurrence suggests that the probiotics would be released in a different part of the intestinal tract whether delivered by one microparticle or another. Microencapsulation conferred greater protective effect to *L. acidophilus* as compared to the free cells. However, the coating of pectin microparticles with whey protein did not confer additional protection to probiotics when exposed to simulated gastrointestinal conditions.

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1. Introduction

Probiotic microorganisms used for human consumption have been a target of great interest from the food industry (Gbassi, Vandamme, Youlou, & Marchioni, 2011). To ensure the role of probiotics during the digestion processes the microorganisms have to reach the site of action and remain at the site long enough to confer a health benefit (Doherty et al., 2011; Heidebach, Först, & Kulozik, 2012; Madureira, Amorim, Gomes, Pintado, & Malcata, 2011).

With the objective of maintaining the viability of probiotics during production, storage and consumption of food to which these microorganisms were added, new technologies have been proposed and among them the microencapsulation seems to be a promising technique. The goal of microencapsulation of probiotics is to protect microorganisms from adverse conditions, enabling the arrival in the intestine at the concentration required to exert its beneficial effect (Kailasapathy, 2002, 2006; Krasaekoot, Bhandari, & Deeth, 2003; Shah, 2000). The probiotic microorganism once in the intestinal system must be completely released by different ways, such as changes in pH, enzymatic activity, time and osmotic strength (Anal & Singh, 2007; Heidebach et al., 2012).

Various materials have been used for microencapsulation of probiotics, such as alginate (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Ding & Shah, 2007; Fávaro-Trindade & Grosso, 2000; Hansen, Allan-Wojtas, Jin, & Paulson, 2002; Kailasapathy & Sureeta, 2004; Mandal, Punija, & Singh, 2006; Shah & Ravula, 2000), κ-carrageenan (Adhikari, Mustapha, & Grün, 2003), cellulose acetate phthalate (Fávaro-Trindade & Grosso, 2002), gelatin (Annan, Borza, & Hansen, 2008; Hsiao, Lian, & Chou, 2004) and pectin (Gerez, Font De Valdez, Gigante, & Grosso, 2012; Oliveira et al., 2007). Whey proteins also have been used by several authors (Doherty et al., 2010, 2011; Kailasapathy & Sureeta, 2004; Picot & Lacroix, 2004) once its use in the microparticles both in matrix and as a coating agent can promote protection for probiotic microorganisms during gastrointestinal transit (Heidebach et al., 2012). Once microparticles produced by ionotropic gelation are porous (Doherty et al., 2011) the coating of particles with different materials has been proposed to increase the protective effect for the delivery of probiotics. In a recent study, Gerez et al. (2012) found that *Lactobacillus rhamnosus* CRL 1505 into pectin particles coated with whey protein improved the survival of the microorganism after exposure to gastric conditions (pH 1.2 and 2.0) when compared to free bacterial cells. In another study, coating sodium alginate particles...
with whey protein increased the survival of *Lactobacillus plantarum* during exposure to simulated gastric juice (pH 1.8) and simulated intestinal juice (pH 6.5) when compared to the microparticle without coating (Gbassi, Vandamme, Ennahar, & Marchioni, 2009).

During the production and characterization of pectin microparticles obtained by ionotropic gelation and coated with whey protein, our research group has demonstrated that protein adsorption on the surface of the microparticles was significantly higher in the particles coated with whey protein without heat treatment (49.2%) than in the particles coated with whey protein heat treated (27.6%). The protein solubility of the microparticles submitted to in vitro gastrointestinal conditions has been affected by both heat treatment and pH levels, since it was greater at a lower pH (1.2) and of the particles coated with whey protein without heat treatment (Souza et al., 2012). Once the whey protein coating protects the microorganism and the heat treatment affects the protein adhered or desorbed from the particle, it is likely that heat treatment before coating may interfere with the protection of the microorganism in the gastrointestinal tract. Based on this hypothesis, the aim of this study was to evaluate whether the coating of pectin microparticles obtained by ionotropic gelation containing *Lactobacillus acidophilus* La5 covered with whey protein heat treated or without heat treatment affects the viability of probiotics when exposed to conditions simulating the passage through the gastrointestinal tract.

### 2. Materials and methods

#### 2.1. Materials

The culture used was probiotic *L. acidophilus* La5 (Christian Hansen Ind. e Com, Valinhos, Brazil). GENU® low methoxyl amidated pectin (CPKelco, Limeira, Brazil) containing 81.3 ± 1.2% galacturonic acid, 30.4 ± 1.6% degree of esterification and 10.4 ± 1.0% degree of amidation (FAO, 2009) was used to pectin solution. Whey protein concentrate (Lacprodan 80, Arla Foods Ingredients, Portenã, CO, Argentina) containing 73.3 ± 0.9% protein and 6.0 ± 0.1% moisture (AOAC, 2006) and common commercial unsalted butter (Laticínios Aviação, São Sebastião do Paraíso, Brazil) were used to produce the particles. To simulate the passage through the gastrointestinal tract the enzymes, mucin (M1778), pepsin (P7012) and pancreatin (P1625) from Sigma-Aldrich Co. (St. Louis, USA) were used. MRS Agar, MRS Broth and GasPak® anaerobic system (Becton, Dickinson and Company, Franklin Lakes, USA) were used for the microbiological analyses.

#### 2.2. Production of cell concentrate of *L. acidophilus* La5

Frozen stock culture of *L. acidophilus* La5 (Christian Hansen Ind. e Com, Valinhos, Brazil) was reactivated twice in MRS broth (2% w/v) at 37 °C for 15 h. The reactivated cells were centrifuged at 8000 g for 10 min at 4 °C. The concentrated cells obtained after centrifugation were resuspended into sterile 0.1% peptone solution and centrifuged under the same conditions, followed by resuspension into peptone solution to obtain a final cell concentration of 9 to 10 log10 CFU/mL. Viability was assayed by pour plating on MRS agar and incubation at 37 °C for 72 h in anaerobic jars using the GasPak® system (De Man, Rogosa, & Sharpe, 1960).

#### 2.3. Microencapsulation and enumeration of *L. acidophilus* La5

The microencapsulation of *L. acidophilus* was performed as described by Gerez et al. (2012) using an aqueous solution of low methoxyl amidated pectin (2% w/w) at pH 4.0 and melted unsalted butter (2% w/w). The mixture was homogenized (Ultra-Turrax Homogenizer, IKA Works Inc., Staufen, Germany) at 14000 rpm for 5 min to obtain an emulsion. The cell concentrate of *L. acidophilus* La5 was added (2% v/v) and homogenized again (6000 rpm for 1 min). The emulsion was atomized in a calcium chloride solution (2% w/v, pH 4.0) under stirring (410 rpm). The microparticles remained in the calcium chloride solution for 30 min to complete gelation and then they were washed (sterile distilled water, pH 4.0) and sieved (pore size 0.125 mm). For coating the microparticles with whey protein by electrostatic interaction, a portion of the pectin microparticles obtained by ionotropic gelation (MPPEC) was immersed for 30 min in a 4% (w/w) solution of whey protein concentrate previously adjusted to pH 4.0 (MPPEC + WP) and the other portion was transferred to a WPC solution previously heat treated at 80 °C for 15 min (MPPEC + WP/HT). The pH 4.0 was chosen because the electrostatic interactions between whey protein concentrate (isoelectric point 4.4–4.5) and pectin (pKa 2.9 approximately) can occur at pH values below 4.5 (Souza et al., 2012) and at lower pH the microorganisms could be affected by the acidity of the solutions during production of the microparticles. The microencapsulation of probiotics was performed at room temperature (25 °C) under aseptic conditions in laminar flow chamber. The materials and solutions used were previously sterilized (121 °C/15 min) with the exception of pectin solution that was filtered using a microbiological filter (AP20 04700 — Millipore, Billerica, USA) with pore sizes from 0.8 to 8 μm.

The viability of *L. acidophilus* was determined by counting viable cells in both cell concentrate and recently produced microparticles. The particles were disintegrated by adding 1 g of microparticles in 9 mL of 2% (w/v) sterile sodium citrate solution at pH 7.0 followed by vigorous stirring for 5 min (Grosso & Favaro-Trindade, 2004; Krasaekoopt, Bhandari, & Deeth, 2004). After disintegration, the microorganisms were released and enumerated. For that, serial dilutions were made in sterile peptone water (0.1% w/v) followed by pour plating on MRS agar using Petri plates, which were incubated at 37 °C for 72 h in anaerobic jar using GasPak® system. After the incubation period the population of probiotics was determined (De Man et al., 1960). The encapsulation yield (YE), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, was calculated as shown in Eq. (1) (Annan et al., 2008):

\[
YE = \frac{N}{N_0} \times 100
\]

where YE is the encapsulation yield, expressed in percentage; N is the number of cells released from the microparticles (log10 CFU/g); and N0 is the number of free cells (log10 CFU/mL) added to the emulsion during microencapsulation process.

#### 2.4. Evaluation of microparticles exposed to simulated gastrointestinal conditions

The simulated gastric and intestinal juices were prepared according to Mozzetti, Gerbino, Fonte De Valdez, and Torino (2009) and Picot and Lacroix (2004), with modifications. The simulated gastric juice (SGJ) was prepared using potassium chloride (1.12 g/L), sodium chloride (2.0 g/L), calcium chloride (0.11 g/L) and potassium phosphate monobasic (0.4 g/L) followed by sterilization at 121 °C for 15 min. Mucin (3.5 g/L) and pepsin (0.26 g/L) were added to SGJ immediately before using and the pH was adjusted to 1.2 or 3.0 by adding 1N HCl. The simulated intestinal juice (SJL) was prepared by adding pancreatin to the SGJ solution to obtain a final concentration of 1.95 g/L and the pH was then adjusted to 7.0 by adding 1N NaHCO3.

The physicochemical evaluation of microparticles and the viability of *L. acidophilus* during simulated gastrointestinal conditions were performed using the methodology adapted from Krasaekoopt et al. (2004) and Gerez et al. (2012). First, either 3 g of the different microparticles (MPPEC, MPPEC + WP and MPPEC + WP/HT) or 3 mL of the concentrate containing free cells of *L. acidophilus* La5 was added separately into test tubes containing 30 mL of simulated gastric juice (SGJ) at pH 1.2 or 3.0. The tubes were incubated at 37 °C in a
metabolic bath with stirring arrangement. Immediately after the addition of the microparticles to the simulated gastric juice (0 min) and after 60 and 120 min exposure, aliquots were removed to evaluate the viability of *L. acidophilus* and the morphology of the particles. A pancreatin solution (final concentration 1.95 g/L) was added to the tubes after 120 min, followed by adjusting pH to 7.0. The tubes were kept in the bath for 300 min and aliquots were removed for further analyses.

The morphology of the microparticles was observed in JENAVAL optical microscope (Carl Zeiss, Oberkochen, Germany) and images were captured by digital camera using the software EDN2-Microscopy Image Processing System. For evaluation of the viability of *L. acidophilus*, 1N NaHCO₃ was added to the aliquots to neutralize the pH, followed by the disintegration of microparticles, dilution and plating as described in Section 2.3.

2.5. Experimental design and statistical analysis

A randomized block with three replications was used. The effect of time of exposure to simulated gastric juice and simulated intestinal juice on the viability of free and microencapsulated *L. acidophilus* was assessed by analysis of variance (ANOVA) and the significant differences between the means for the values obtained were evaluated by Tukey's test at 5% level of significance. The data were analyzed using the software STATISTICA 7.0 (StatSoft, Inc, Tulsa, USA).

3. Results and discussion

3.1. Microencapsulation of *L. acidophilus*

Microencapsulation of *L. acidophilus* by ionotropic gelation using pectin as wall material (MPREC) followed by coating with whey protein heat treated or without heat treatment (MPREC+WP and MREC+WP) resulted in a high microencapsulation yield, which was on average 84.35±0.60% and not significantly different between the particles. The average count of *L. acidophilus* on the different particles was 8.31±0.24 log₁₀ CFU/g. The comparison between encapsulation yields reported in literature is complicated by the wide range of microorganisms studied, encapsulating techniques and wall material used. For instance, Corbo, Bevilacqua, and Sinigaglia (2011) found similar encapsulation yield (83.33%) to that found in this study using ionotropic gelation and alginate for encapsulation of *L. rhamnosus*. On the other hand, Chávarri et al. (2010) found yields varying from 19.5 to 40.2% for encapsulation of *Lactobacillus gasseri* and *Bifidobacterium bifidum* using the same encapsulation technique with chitosan coating. Using spray drying method for microencapsulation of *Bifidobacterium breve* and *Bifidobacterium longum*, Picot and Lacroix (2004) obtained yields ranging from 0.03 to 25.67%. The low encapsulation yield found by these authors may be related to the sensitivity of microorganisms to high process temperatures, since the encapsulation by spray drying was carried out using outlet air temperature of 80 °C.

The high encapsulation yield observed in our study (84.35%) may be due to the natural resistance of the microorganism and the conditions of encapsulation, which was carried out at room temperature (25 °C), without using organic solvents and at pH 4.0, which favors the interaction between the pectin and whey proteins, as previously demonstrated by Souza et al. (2012).

3.2. Evaluation of microparticles exposed to simulated gastrointestinal conditions

The exposure to a series of conditions that simulate the passage through the gastrointestinal tract showed that the pectin microparticles (MPREC) remained intact in both simulated gastric juice at pH 1.2 and 3.0 for 120 min and in simulated intestinal juice at pH 7.0 for 300 min. (Figs. 1 and 2). Although the particles coated with whey protein heat treated or without heat treatment (MPREC+WP/H or MREC+WP) have remained intact during exposure to low pH (pH 1.2 and 3.0) in simulated gastric juice, they were completely degraded after exposure to pH 7.0 in simulated intestinal juice. Figs. 3 and 4 show the morphological behavior of MREC+WP/H in SGJ at pH 1.2 and 3.0 and in SJ at pH 7.0. The disintegration of microparticles containing probiotic bacteria enables their release so that it can colonize the intestinal tract and confer benefits on the host (Buddington, 2009). Therefore, it is possible that the releasing of probiotics from coated microparticles (MREC+WP+HT and MREC+WP/H) occurs either in small or large intestines whose pH varies from 6.15 to 7.88 and 5.20 to 7.02, respectively (Cook, Tzortzis, Charalampopoulos, & Khutoryanskaya, 2012). On the other hand, the encapsulated probiotics would probably be released in the colon, which offers near neutral pH and pectic enzymes (Liū, Fishman, Kost, & Hicks, 2003). Disintegration of coated whey protein microparticles in simulated gastrointestinal conditions was observed by several authors (Doherty et al., 2011; Gbassi et al., 2009, 2011; Picot & Lacroix, 2004).

Concerning the viability of *L. acidophilus*, it reduced during exposure to simulated gastrointestinal conditions for all treatments, as can be seen in Table 1. However, the free cell count showed a reduction level of 3.54 log units after exposure to simulated gastric juice (pH 3.0) and simulated intestinal juice (pH 7.0), while the encapsulated probiotic bacteria showed reduction levels of 1.51, 1.59 and 1.67 log cycles for MREC, MREC+WP and MREC+WP/H, respectively. Thus, the microencapsulation conferred *L. acidophilus* protection during exposure to simulated gastric juice at pH 3.0. Moreover, coating of microparticles (MPREC) with whey protein heat treated or without heat treatment (MPREC+WP/H or MREC+WP) did not confer additional protection to *L. acidophilus*, once it did not prevent diffusion of acidic groups and enzymes into the particles (Doherty et al., 2011), affecting the viability of probiotics before (MPREC) and after coating (MPREC+WP/H and MREC+WP) in a similar way. In more drastic pH (pH 1.2) microencapsulation did not provide any protection to *L. acidophilus* (Table 2). After 120 min exposure to simulated gastric juice at pH 1.2 the viability of free cells as well as encapsulated (MPREC, MREC+WP and MREC+WP/H) was below the detection limit (<2 log₁₀ CFU/mL).

Favorable and unfavorable outcomes are described in the literature regarding the efficacy of the coating of particles aiming at improving the probiotic viability in the intestinal tract. The controversial results concerning the protection given by microencapsulation may be due to the conditions of the study, such as natural resistance of the microorganism, different pH values, presence or absence of enzymes and different wall materials. As described by Krasekoop et al. (2004) chitosan coating provided better protection to *L. acidophilus* 547 and *Lactobacillus casei* as compared to uncoated alginate particles. However, the particle was not effective in protecting *B. bifidum* ATCC 194 using the same conditions defined by the authors (pH 1.55 for 2 h in the absence of enzymes). Coating alginate particles with whey protein conferred greater protection to *L. plantarum* when exposed both to simulated gastric juice (pH 1.8 in the presence of pepsin) and simulated intestinal juice (pH 6.5, in the presence of pancreatic, trypsin and bile salts) when compared to the particles without coating (Gbassi et al., 2009). Coating pectin particles with whey protein also conferred greater protection to *L. rhamnosus* CRL 1505 when exposed to acidic condition (pH 2.0 in the presence of mucin and pepsin) as compared to free microorganisms (Gerez et al., 2012), but the survival of the microorganisms in the uncoated particles was not evaluated.

The results presented in this work corroborate other studies in literature that show the efficiency of microencapsulation in protecting probiotic microorganisms during exposure to gastrointestinal conditions (Gbassi et al., 2009; Gerez et al., 2012; Krasekoop et al., 2004). Some factors such as microorganisms studied, different encapsulation
Fig. 1. Morphology of pectin microparticles obtained by ionotropic gelation during sequential exposure to both simulated gastric juice (SGJ) at pH 1.2 for 120 min and simulated intestinal juice (SIJ) at pH 7.0 for 300 min. A: Immediately after exposure to SGJ at pH 1.2; B: 60 min exposure to SGJ at pH 1.2; C: 120 min exposure to SGJ at pH 1.2; D: 300 min exposure to SIJ at pH 7.0. Bar = 75 μm.

Fig. 2. Morphology of pectin microparticles obtained by ionotropic gelation during sequential exposure to both simulated gastric juice (SGJ) at pH 3.0 for 120 min and simulated intestinal juice (SIJ) at pH 7.0 for 300 min. A: Immediately after exposure to SGJ at pH 3.0; B: 60 min exposure to SGJ at pH 3.0; C: 120 min exposure to SGJ at pH 3.0; D: 300 min exposure to SIJ at pH 7.0. Bar = 75 μm.
Fig. 3. Morphology of pectin microparticles obtained by ionotropic gelation coated with whey protein heat treated during sequential exposure to both simulated gastric juice (SGJ) at pH 1.2 for 120 min and simulated intestinal juice (SIJ) at pH 7.0 for 300 min. A: Immediately after exposure to SGJ at pH 1.2; B: 60 min exposure to SGJ at pH 1.2; C: 120 min exposure to SGJ at pH 1.2; D: 300 min exposure to SIJ at pH 7.0. Bar = 50 μm.

Fig. 4. Morphology of pectin microparticles obtained by ionotropic gelation coated with whey protein heat treated during sequential exposure to both simulated gastric juice (SGJ) at pH 3.0 for 120 min and simulated intestinal juice (SIJ) at pH 7.0 for 300 min. A: Immediately after exposure to SGJ at pH 3.0; B: 60 min exposure to SGJ at pH 3.0; C: 120 min exposure to SGJ at pH 3.0; D: 300 min exposure to SIJ at pH 7.0. Bar = 50 μm.
conditions and methods used to evaluate the encapsulation efficiency (pH, presence or absence of enzymes and comparing between coating conditions) have limited the efforts in finding the best coating technique for maintaining the viability of probiotic bacteria during the passage through the gastrointestinal tract.

4. Conclusions

Microencapsulation of L. acidophilus La5 by ionotropic gelation using pectin as wall material followed by covering with whey protein showed high encapsulation yield and positively affected the viability of the microorganism when exposed to conditions simulating the transit through the gastrointestinal tract. The coating of the particle with whey protein did not confer additional protection to probiotic; however, it affected the rupture of microparticles under conditions which simulate the gastrointestinal tract, which would enable the release of microorganisms in different parts of theIntestine.

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