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Evaluation of the viability and the preservation of the functionality of microencapsulated *Lactobacillus paracasei* BGP1 and *Lactobacillus rhamnosus* 64 in lipid particles coated by polymer electrostatic interaction

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ABSTRACT

This study aimed to evaluate the resistance, viability, and functionality of two strains of probiotics immobilized in solid lipid microparticles covered by a complex of gelatin and gum Arabic. Microcapsules were evaluated regarding their size, morphology, and resistance under stress conditions. Encapsulated microorganisms were evaluated concerning during storage for 120 days. Additionally, the effect of encapsulation on the functionality of the microorganisms was investigated using an *in vivo* assay. Microcapsules had sizes around 80 μm and extreme pH and temperature of 50 °C destabilized them. Encapsulation improved the stability of these microorganisms in the presence of salt and in gastrointestinal conditions. Encapsulated microorganisms maintained their viability during storage and the dosage of S-IgA and cytokines (IL-2, IL-6, IL-10, and TNF-α) in mice indicated that encapsulated microorganisms maintained their functionality. Therefore, the microencapsulation technique may be promising for the improvement of the viability of probiotics under adverse conditions without compromising their immunomodulating capacity.

1. Introduction

Probiotics are living microorganisms that when administered in adequate amounts may result in health benefits to those that consume them (FAO/WHO, 2002). However, for these benefits to be obtained, it is important that the microorganisms arrive alive in the intestine and consumed in a concentration ranging from 10^6 to 10^8 CUF/g of food. The dose-response may vary depending on the expected benefit (Ouwehand, 2017).

In order to ensure the survival of probiotics during food processing and storage, as well as during their passage through the gastrointestinal tract, and with the aim of guaranteeing their release under specific conditions, our research group has worked on the development and use of microencapsulation techniques (Fávaro-Trindade & Grosso, 2002; Gbassi & Vandamme, 2012; Okuro, Thomazini, Balieiro, Liberal, & Fávaro-Trindade, 2013; Oliveira et al., 2007a, 2007b; Pedroso, Dogenski, Thomazini, Heinemann, & Favaro-Trindade, 2013; Pedroso, Thomazini, Heinemann, & Favaro-Trindade, 2012; Trindade & Grosso, 2000).

The microencapsulation of probiotics consists of the incorporation of bacteria in a matrix or membrane capable of isolating them from the external environment, providing protection and enabling controlled release (Rathore, Desai, Liew, Chan, & Heng, 2013). Several techniques have been used to encapsulate probiotics, including spray drying, spray chilling/spray congealing, fluidized bed, ionic gelation, and complex coacervation (Favaro-Trindade, 2011; Rokka & Rantamäki, 2010).

The microencapsulation by complex coacervation involves the interaction of oppositely charged polymers, usually proteins and

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polysaccharides, which are mixed in a solution in order to form a coating around the component that will be encapsulated (Rokka & Rantamäki, 2010).

Complex coacervation has been used by other researchers to microencapsulate probiotics using different polymers (Oliveira et al., 2007a, 2007b; Bosnea, Moschakis, & Biliaderis, 2014; Caetano-Silva et al., 2015). Since this technique does not require the use of organic solvents or high temperatures, it may be an interesting alternative for the encapsulation of probiotic cells. Nonetheless, although the microencapsulation of probiotics has been successful, even if this technology can increase the probiotic survival rate, it does not necessarily imply in the preservation of functionality (de Vos, Faas, Spasojevic, & Sikkema, 2010).

Few studies have evaluated the effect of encapsulation or other technological processes on the preservation of the functionality of probiotic strains, which we intend to assess in this study. Recently, Zacarías et al. (2017) evaluated the effect of spray drying or lyophilization on the preservation of the functionality of *Bifidobacterium lactis* INL1 using the dosage of IgA, IL-10, and IFN-γ, stating that these processes negatively influenced the immunological stimulation ability of the strain. Lavari, Burns, Páez, Reinheimer, and Vinderola (2017) found that spray drying *L. rhamnosus* 64 modified its immunomodulating capacity in healthy animals, particularly considering the cytokine profile. Bajracharya et al. (2012) evaluated the effect of microencapsulating *L. salivarus* 29 in microcapsules of alginate/chitosan/alginate on cytokine induction *in vitro* and found that the free bacteria presented a greater ability of stimulating TNF-α and IL-10 in comparison to the microencapsulated bacteria.

In view of what was presented above, the purpose of this study was to microencapsulate *L. paracasei* BGP1 and *L. rhamnosus* 64 strains in lipid particles coated by electrostatic interaction between gelatin and gum Arabic, as well as to characterize the microcapsules obtained and to evaluate the effectiveness of the encapsulation process in improving the viability of the strains under technological stress conditions, passage through the gastrointestinal tract, and storage. The preservation of the immunomodulating capacity of the microencapsulated strains was also tested by means of *in vitro* tests using the Secretory IgA (S-IgA) and cytokine dosage in mice as parameters.

2. Material and methods

2.1. Microorganisms

L. rhamnosus 64 was isolated from newborn human feces (Vinderola et al., 2008) and *L. paracasei* BGP1 was obtained from a local provider. Vegetable fat TRI-CS48 (with melting point at 43 °C) was donated by Triangulo Alimentos (Itápolis, Brazil). The polymers gelatin (pI 4.5), and gum Arabic were acquired from Gelnex (Itá, Brazil) and Dinâmica Química Contemporânea (Diadema, Brazil), respectively.

2.2. Methods

2.2.1. Determination of the ideal pH for the production of microcapsules

Five pH values (3.0, 3.5, 4.0, 4.5 and 5.0) were evaluated in order to determine the ideal pH for the production of microcapsules. The selection of the pH was performed considering the development of microcapsules with a typical morphology for this technique – spherical microcapsules with an apparent nucleus (Santos, Bozza, Thomazini, & Favaro-Trindade, 2015), and considering the process yield in terms of the decantation efficiency of the microcapsules after 24 h of storage at 7 ± 1 °C by means of visual evaluation. This determination was based on the description presented by Siow and Ong (2013).

2.2.2. Production of microcapsules loaded with L. paracasei BGP1 and L. rhamnosus 64

The microcapsules loaded with *L. paracasei* BGP1 and *L. rhamnosus* 64 were prepared using the complex coacervation technique based on the method described by Comunian et al. (2013), with modifications. Initially, the obtained vegetable fat was melted 7 °C above its melting point (43 °C). The inoculum obtained by the centrifugation of the microorganism grown in MRS (16 h, 37 °C) was added to the molten vegetable fat at a concentration of 10^9 CFU/g. Following this step, a solution of gelatin (2.5% m/v) was added to the microorganism and vegetable fat mixture in order to obtain an emulsion (O/A). This emulsion was produced by homogenization using an Ultra-Turrax at 7000 rpm for 90 s. Subsequently, an aqueous solution of gum Arabic (2.5% w/v) was added to the emulsion under constant magnetic stirring. In order to promote the coacervation process, the pH of the mixture was adjusted to 4.5 using a NaOH 4 M solution, this value was determined by the test described in the next section. Up to this step, the whole process was conducted while maintaining the mixture under heating at 50 °C, preventing the solidification of the vegetable fat, as well as the gelation of the gelatin. After adjusting the pH, the temperature of the mixture was reduced to 10 °C using an ice bath. The coacervated material was maintained at 7 °C during 24 h for the decantation of the coacervated particles. Lastly, the microparticles were frozen and dehydrated by sublimation in a lyophilizer (Terroni, São Carlos, Brazil) for 24 h at 1–0.1 kPa pressure, condensation temperature of −20 °C and final temperature of 30 °C. After the dehydration process, the microcapsules were stored in glass jars, protected from light and maintained in the presence of oxygen at 7 ± 1 and 25 ± 1 °C.

2.2.3. Morphology and particle size

The microcapsules were characterized by optical microscopy and scanning electron microscopy (SEM) using an optical microscope (BIO3 – Bel Photonics - MB, Italy) and Tabletop Microscope TM 3000 (Hitachi, Tokyo, Japan), respectively. In addition, the microcapsules were characterized by confocal microscopy (Leica, DMIRBE, Nova Yorque, USA) and nile red colorant (Sigma-Aldrich, St. Louis, EUA) was applied in the fat phase of the microcapsules (5% m/m), according to Santos et al. (2015). The average size of the microparticles was determined by laser diffraction (Shimadzu Sald-201V particle size analyzer, Kyoto, Japan).

2.2.4. pH effect on the stability of the microcapsules

The stability of the microcapsules was evaluated considering five pH values, 1.5, 3.0, 4.5, 7.5, and 9.0. In order to do so, the microcapsules were maintained in distilled water for 30 min with the respective pH values adjusted with HCl or NaOH solutions. After reaching the established time, the microcapsules were submitted to morphological analysis by means of optical microscopy.

2.2.5. Temperature effect on the stability of the microcapsules

The thermal stability of the microcapsules was evaluated considering three temperatures, 40, 50, and 60 °C. The microcapsules suspended in distilled water were subjected to heating at the defined temperatures for 2 min and were then evaluated by optical microscopy.

2.2.6. Sensitivity of microorganisms to the encapsulation process

In order to evaluate the sensitivity of both strains to the encapsulation process by complex coacervation, the enumeration of microorganisms was carried out in three moments: (a) in the cellular mass added to the vegetable fat; (b) in the wet microcapsules after the decanting process; and (c) in the dehydrated microcapsules. In order to determine the microorganism content of the microcapsules, 2 g of microcapsules were weighed (dry weight) and heated at 50 °C for 30 s for the disruption and release of the microorganisms, followed by serial dilution in 2% sodium citrate solution (w/v), plating on MRS agar and incubation at 37 °C for 48 h under aerobic condition.

2.2.7. Sensitivity of microorganisms to the homogenization process using an Ultra-Turrax

In order to evaluate the sensitivity of the microorganisms to the

homogenization process using an Ultra-Turrax, the strains cultured in MRS broth overnight (16 h) were washed twice (6000 rpm/6 min/7 °C) and resuspended (2 ml of cell dispersion) in 10 ml of phosphate buffered saline (0.1 M, pH 7.2). The suspension was homogenized in an Ultra-Turrax for 90 s at 5500, 7000, and 8500 rpm. The enumeration of microorganisms was conducted before and after the homogenization process.

2.2.8. Tolerance of free or encapsulated microorganisms to temperature

The study of the tolerance of free or encapsulated microorganisms to temperature was conducted based on the methodology described by Bosnea et al. (2014), with modifications. The microorganism mass produced after overnight incubation (16 h) in MRS broth was washed twice (6000 rpm/6 min/7 °C) and resuspended (2 ml cell dispersion) in a phosphate buffered saline (0.1 M, pH 7.2). Microcapsules loaded with microorganisms (2 g of microcapsules) were also suspended in phosphate buffered saline for analysis. Free and encapsulated microorganisms were exposed to temperatures of 40, 50, and 60 °C for 5 min. The enumeration of microorganisms was conducted before and after thermal exposure.

2.2.9. Tolerance of free or encapsulated microorganisms to salinity

The tolerance of free and microencapsulated microorganisms to three saline solution concentrations was determined according to the methodology described by Bosnea et al. (2014), with modifications. Analogously to the thermal tolerance, the microorganism mass produced after overnight incubation (16 h) in MRS broth was washed twice (6000 rpm/6 min/7 °C) and resuspended (2 ml cell dispersion) in a phosphate buffered saline (0.1 M, pH 7.2). The microcapsules loaded with microorganisms (2 g of microcapsules) were also suspended in a phosphate buffered saline. Free and encapsulated microorganisms were exposed to the NaCl solution at 1, 2.5, or 5.0% (w/v) for 2 h at 37 °C. Tolerance was determined by the difference in the enumeration of viable microorganisms before and after exposure.

2.2.10. Tolerance of free and encapsulated microorganisms to simulated gastrointestinal fluids

The *in vitro* evaluation of the resistance of free microorganisms in simulated gastric fluid (SGF) and simulated intestinal fluids (SIF) was conducted according to the method described by Gbassi, Vandamme, Ennahar, and Marchioni (2009). The SGF was prepared using 9 g/L NaCl (Synth, Diadema, Brazil), 3 g/L pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, USA) in distilled water and pH adjusted to 1.8 with HCl. The SIF was prepared using 9 g/L NaCl (Synth, Diadema, Brazil), 10 g/L pancreatin (Sigma-Aldrich, St. Louis, USA), 9 g/L bovine trypsin (Sigma-Aldrich, St. Louis, USA), and 3 g/L bile salts - Oxgall (Difco, Hampshire, UK) in distilled water and pH adjusted to 6.5 with NaOH. The microorganisms were inoculated into the SGF and maintained at 37 °C under agitation. The enumeration of viable cells was performed with MRS after 0, 60, and 120 min. The remaining SGF analysis material was inoculated into SIF, followed by adequate pH control and adjustment and enumeration of viable cells with MRS after 0, 90, and 180 min.

2.2.11. Evaluation of the viability of microencapsulated microorganisms during storage

After the dehydration process, the microcapsules were stored in glass jars, protected from light, and maintained in the presence of oxygen at 7 ± 1 and 25 ± 1 °C. The viability of the microorganisms was evaluated by the enumeration of viable cells after 0, 30, 60, 90, and 120 days.

2.2.12. Evaluation of the preservation of the immunomodulating capacity of free and microencapsulated L. Paracasei BGP1 and L. Rhamnosus 64

2.2.12.1. Animal handling. Sixteen male BALB/c mice with five to six weeks of age were used in this study. The animals were kept for 1 week in the laboratory of the Instituto de Lactología Industrial (Santa Fe –Argentina) for acclimatization at a temperature of 21 ± 1 °C, with a 12 h light-dark cycle, total air renewal rates of 20 changes/h and *ad libitum* access to water and a conventional balanced diet for rodents (Cooperación, Buenos Aires, Argentina). The project was approved by the Ethics and Research Committee of the Universidad Nacional del Litoral prior to the beginning of the study.

2.2.12.2. Experimental design for the administration procedure. In order to evaluate the effect of encapsulation on the preservation of the immunomodulatory capacity of the two strains studied, the mice were divided into 4 groups with 4 animals per group. Group 1: received daily doses of free *L. paracasei BGP1*; Group 2: received daily doses of free *L. rhamnosus 64*; Group: 3 received daily doses of microcapsules loaded with *L. paracasei BGP1*; and Group 4: received daily doses of microcapsules loaded with *L. rhamnosus 64*. The mice received daily doses of 0.3 ml containing the free or encapsulated microorganisms by gavage for 10 consecutive days.

The daily doses received by each animal contained 10^7 CFU/mL of lactobacilli, regardless of the form in which the probiotic was presented (free or encapsulated). For administration, free or microencapsulated microorganisms were suspended in sterile saline solution. After 10 days of probiotic administration, the mice were anesthetized with ketamine, xylazine, and acepromazine and sacrificed by cervical dislocation. Different tissues were extracted for the analyses described in the following sections.

2.2.12.3. Determination of secretory immunoglobulin A in the intestinal content. The determination of the level of secretory IgA was done by the Capture-ELISA method according to the methodology described by Rodrigues et al. (2000) and Souza et al. (2012). The intestinal content used for the analysis was obtained after the removal of the intestine through cuts in the gastroduodenal and ileocecal junction. The intestinal content was weighed and washed with saline phosphate buffer (pH 7.2). Protease inhibitor 1% (v/v) was added to the PBS. After centrifugation (2000*g*, 30 min, 4 °C), the supernatant was collected and stored at −20 °C. The concentration of the total secretory IgA was determined using a purified IgA standard. The reading at 492 nm was done using an ELISA plate reader (Thermo Scientific Multiskan Spectrum, Wilmington, USA) and the results were expressed as μg S-IgA/g intestinal content, calculated from the standard curve.

2.2.12.4. Determination of cytokines in the gut. To determine cytokines, sample preparation was performed according to the methodology described by Zacarías et al. (2017). After tissue extraction, small and large intestine samples were maintained frozen (−70 °C). Initially, the samples were homogenized in PBS buffer containing 1% (v v-1) of an anti-protease cocktail (Sigma), 10 mmol L-1 EDTA (Sigma) and 0.05% (v v-1) of Tween 20 (Sigma) at a 1 mL PBS to 100 mg tissue ratio. The samples were centrifuged (9500*g*, 10 min, 4oC) and the supernatants were collected and maintained frozen until cytokine determinations (TNF, IL-10, IL-6 and IL-2). Cytokines were dosed by using commercial ELISA kits following the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA).

2.3. Statistical analysis

With the exception of the animal tests (performed once), all other tests were performed at least in duplicate or triplicate. Data were analyzed using Analysis of Variance (ANOVA) followed by the Tukey's multiple comparison tests when necessary. Analyses were carried out using GraphPad Prism software version 6.0. Means were considered significantly different when $p < 0.05$.

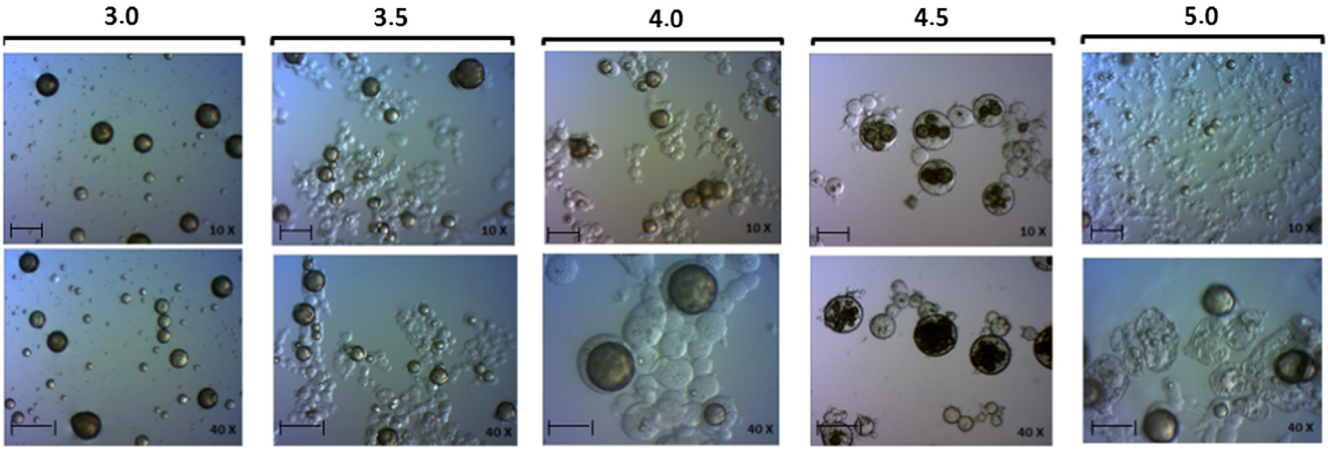

Fig. 1. Micrographs obtained by optical microscopy of coacervates produced with different pH values: 3.0, 3.5, 4.0, 4.5, and 5.0.

3. Results and discussion

3.1. Determination of the ideal pH for the production of microcapsules

During the complex coacervation process, the pH represents a crucial factor for the successful production of microcapsules, influencing the process yield. This occurs because the pH affects the development of the protein-carbohydrate complex due to its influence on the degree of ionization of both the protein's (amino group) and the carbohydrate's (carboxyl group) functional groups.

Fig. 1 presents the images captured by the optical microscopy of the material obtained by complex coacervation in which different pH values were evaluated. With the exception of the treatments in which the pH was adjusted to 3.0 and 5.0, microcapsules with typical characteristics of a complex coacervation technique were obtained, that is, spherical microcapsules with well-delimited edges and apparent nuclei (Nori et al., 2011; Prata, Zanin, Ré, & Grosso, 2008; Rocha-Selmi, Theodoro, Thomazini, Bolini, & Favaro-Trindade, 2013), which can be clearly observed in Fig. 3A. In the micrographs regarding the material produced at pH 3.0, only fat globules can be observed. In turn, in the micrographs concerning the material produced at pH 5.0, the development of the protein-polysaccharide complex and some fat globules internalized in the complex can be observed, although without a definite form, indicating that the encapsulation process did not occur. Among the pH values tested, the coacervate produced at pH 4.5 presented the expected morphological characteristics.

Fig. 2 shows the results of the evaluation of the decantation efficiency of the coacervate 24 h after the production of the microcapsules. The identification of the phase separation was not possible when the coacervate was prepared at pH 3.0. In the other pH values (3.5, 4.0, 4.5, 5.0) the phase separation is clear. It is valid to highlight that the tube containing the coacervate prepared at pH 4.5 was the one that presented the most visually clear aqueous phase. Considering the results obtained, particularly regarding morphology and decantation efficiency, pH 4.5 was chosen to produce microcapsules by complex coacervation in this study.

Fig. 2. Evaluation of process yield by the sedimentation efficiency of the coacervates produced with different pH values (3.0, 3.5, 4.0, 4.5, 5.0).

3.2. Effect of the pH and the temperature on capsule stability

Considering that the selection of the ideal pH value is a crucial and limiting factor for the production of microcapsules by the complex coacervation technique, evaluation of the pH effect on capsule stability is also very important. As stated by Comunian et al. (2016), the complex coacervation process involves interactions that depend on specific pH values, however, these attractive forces, called electrostatic bonds, are very weak and can be altered or eliminated by changes in pH. Thus, pH values may influence the process of releasing the content of the capsule in a desirable or an undesirable manner. Furthermore, pH values may also interfere with the protection of probiotics under the adverse conditions of the gastrointestinal tract, which includes environments with very low pH values.

Fig. 4-I presents the images obtained by the optical microscopy of the microcapsules loaded with lactobacilli after exposure to solutions with different pH values. Considering the typical morphological characteristics of the microcapsules, it is possible to observe that the integrity of the microcapsules was maintained after exposure to different pH conditions. However, it is also possible to verify that the delimitations of the microcapsules were considerably weakened as the pH values became extreme and distanced themselves from the pH of 4.5, which was used for the production of the microcapsules. Considering the results obtained, greater attention should be given to the analysis of the tolerance to gastrointestinal fluids, discussed in a subsequent topic.

Fig. 4-II shows the micrographs obtained by optical and confocal microscopy of the microcapsules after being exposed to three

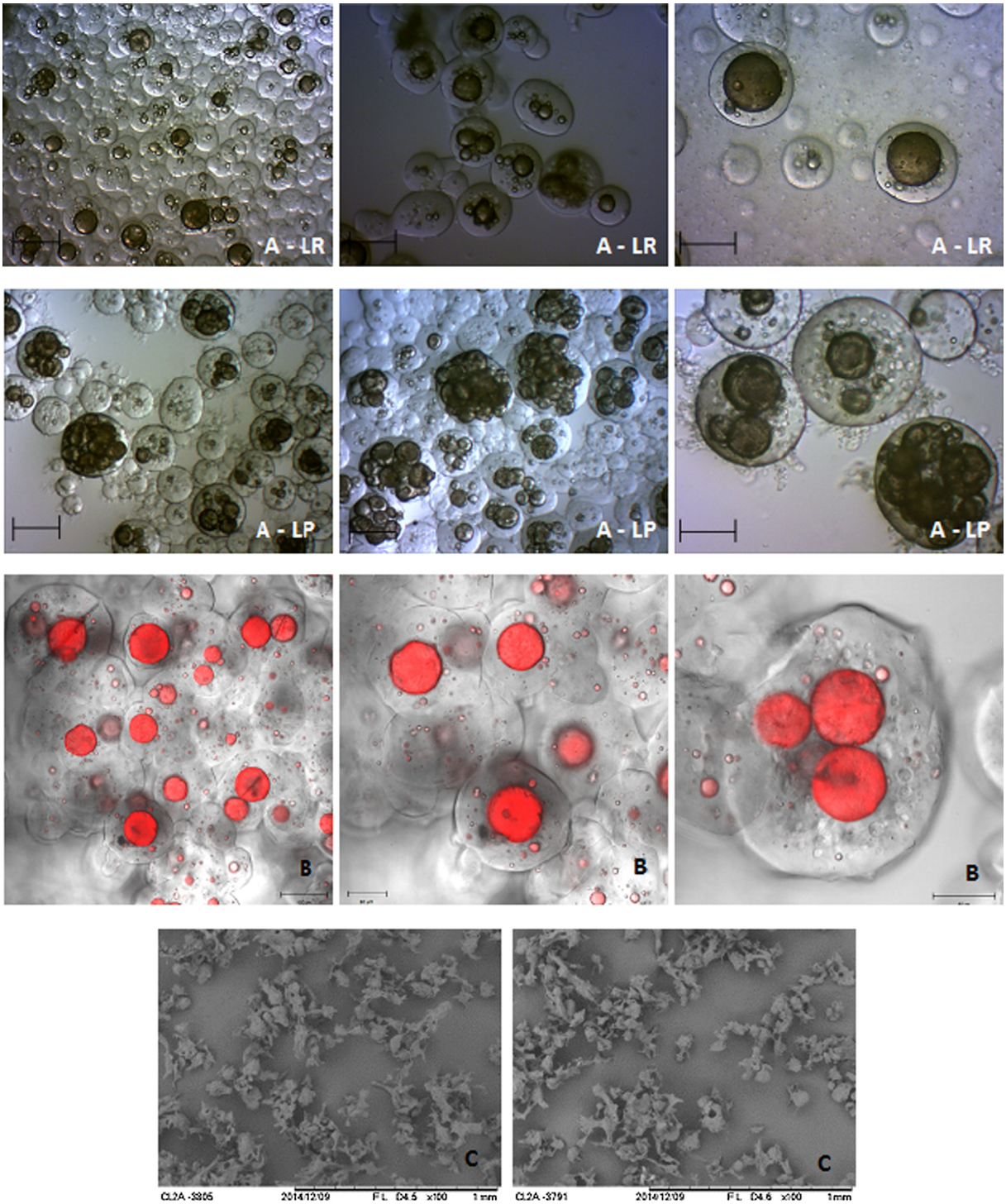

Fig. 3. Micrographs obtained by optical microscopy (A) - LR: *L. rhamnosus* 64 and LP: *L. paracasei* BGP1, confocal microscopy (B) and scanning electron microscopy (C) of microcapsules loaded with lactobacilli produced by complex coacervation.

temperature values, 40, 45, and 50 °C for 2 min. The microcapsules did not undergo morphological alterations after exposure to the temperatures of 40 and 45 °C, which is observed by the well-delimited edges and apparent nuclei. However, the microcapsules were totally altered when heated to 50 °C, which is verified by the presence of small fat globules that indicate their disruption. This may have been promoted by the destabilization of the protein-polysaccharide complex, as a function of the molecule agitation.

3.3. Resistance of microorganisms to the encapsulation process

The results regarding the resistance of microorganisms to the complex coacervation encapsulation process can be visualized in Fig. 5-I. The population of *L. rhamnosus 64* was not reduced during the process so that its initial count of 10^9 CUF/g was maintained after encapsulation. However, the population of *L. paracasei BGP1* suffered a reduction in the order of 1 logarithmic cycle after the production of the microcapsules. After collecting the microcapsules, the enumeration of the microorganisms present in the supernatant of the material prepared in

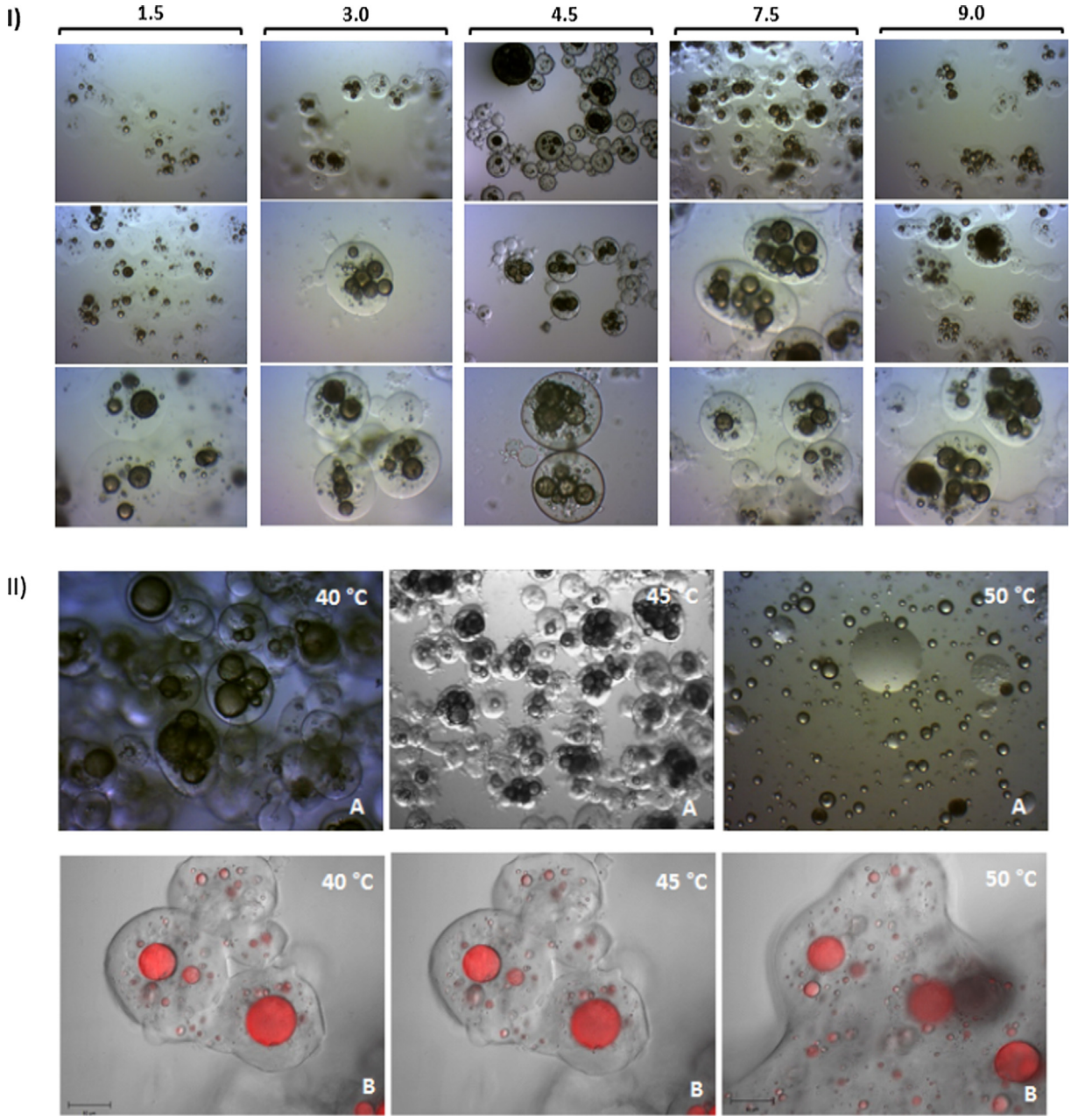

Fig. 4. Micrographs obtained by optical microscopy of microcapsules produced by complex coacervation with different adjustment pH values (1,5, 3,0, 4,5, 7,5 and 9,0) in order to determine the ideal pH value for the process (I). Micrographs of microcapsules loaded with probiotics submitted to different temperatures (40, 45, and 50 °C) were captured by optical microscopy (A) and confocal microscopy (B) in order to evaluate the effect of the temperature on the structural stability of the microcapsules (II).

the coacervation did not reach 2.00 log CFU/mL for *L. rhamnosus 64*, but was 3.24 log CFU/mL for *L. paracasei BGP1*, suggesting that differences in concentrations after capsule production result from the presence of bacteria in the aqueous phase in which the microcapsules were formed.

Once again, it is opportune to explore the hydrophobicity profile of the cell walls of both microorganisms. *L. rhamnosus* 64 presented a more hydrophobic cell wall than *L. paracasei* BGP1, which could justify the higher retention in the lipid particle and, therefore, the tendency to pass into the aqueous environment during the microcapsule development process. These results can be considered satisfactory when establishing a comparison with the ones obtained in a study developed by Silva et al. (2018), in which the authors encapsulated *Lactobacillus acidophilus* LA3 (LA) and *Bifidobacterium animalis* subsp. *lactis* BLC1 (BLC) and determined the loss of 2 log cycles in the supernatant water using a similar technique to the one in this present study.

3.4. Resistance of microorganisms to the homogenization process using an Ultra-Turrax

The use of an Ultra-Turrax for homogenization during the microencapsulation process represents an important risk factor since the homogenization occurs by means of sheer tension and there is the possibility of physically damaging the bacterial cells.

No significant reduction in the enumeration of *L. rhamnosus* 64 and *L. paracasei* BGP1 viable cells was verified when submitted to homogenization using an Ultra-Turrax at 5500 and 7000 rpm for 90 s ($p < 0.05$). Nevertheless, a reduction in the order of 1 logarithmic cycle was verified regardless of the evaluated strain when the evaluated speed was 8500 rpm for 90 s ($p < 0.05$). These results corroborated the

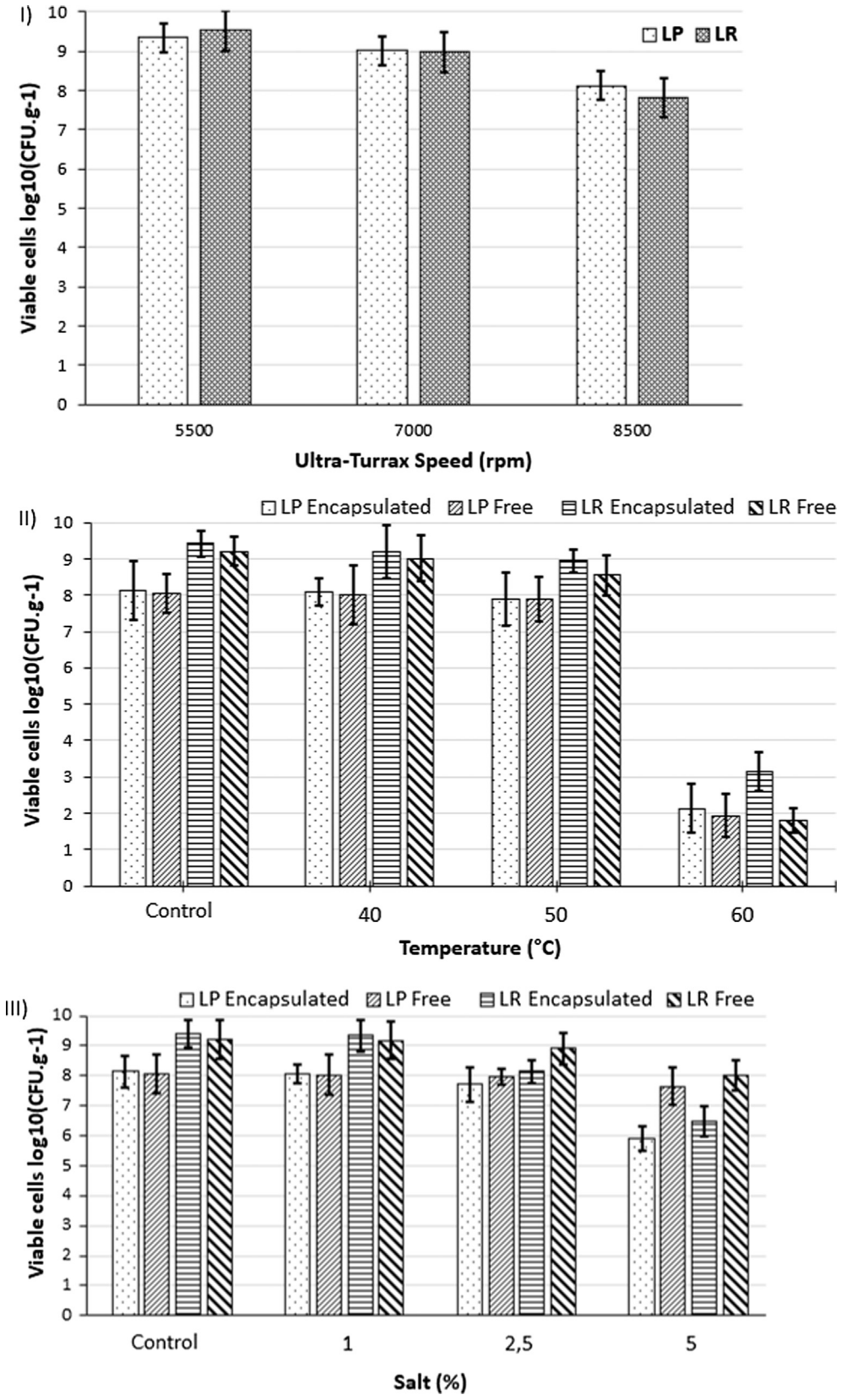

Fig. 5. Resistance of *L. paracasei* BGP1 (LP) and *L. rhamnosus* 64 (LR) to the process of homogenization with an Ultra-Turrax at different speeds (5500, 7000, and 8500 rpm) (I); Resistance of free and encapsulated *L. paracasei* BGP1 (LP) and *L. rhamnosus* 64 (LR) after exposure to different temperatures (40, 50, and 60 °C) for 5 min (II); After exposure to different concentrations of NaCl (1, 2.5, and 5%) for 120 min (III).

ones obtained by Capela, Hay, and Shah (2007) and Ding and Shah (2009), indicating that the increase in homogenization velocity has a deleterious effect on the bacteria. Furthermore, these results supported the choice of the rotation speed/time binomial at 7000 rpm for 90 s for the process of obtaining the microcapsules considering the preservation of the integrity of the microorganisms.

3.5. Tolerance of free and encapsulated microorganisms to temperature and salinity

In addition to the possibility of increasing the viability of probiotic microorganisms during the storage period and of promoting protection against the adverse effects found in the gastrointestinal tract, microencapsulation may be useful in protecting probiotic cells during food processing, especially considering the deleterious effects associated with heat treatment, such as cell damage.

The effect of temperature on the two strains of lactobacilli is shown in Fig. 5-II. The encapsulation was only effective in the protection of *L. rhamnosus* 64 (LR) when exposed to the temperature of 60 °C, avoiding the reduction of 1,33 log CFU/mL considering the result obtained for free cells ($p < 0.05$). In all other conditions, no significant differences were found when comparing the results of free and encapsulated cells ($p > 0.05$).

The impact of the increase in temperature was only significant when the exposure temperature was 60 °C. The reductions were of 6.00 and 6.12 log CFU/mL for encapsulated and free *L. paracasei* BGP1, respectively, and of 7.41 and 6.27 log CFU/mL for encapsulated and free *L. rhamnosus* 64, respectively.

Considering the results obtained in this study, the encapsulation in lipid particles covered by gelatin and gum Arabic did not promote an improvement in the thermal tolerance of the two strains of evaluated lactobacilli. These results could have been different if the exposure time had been prolonged, since Ann et al. (2007) also found no significant differences in viability between free or encapsulated *L. acidophilus* ATCC 43,121 after 60 min of exposure at 55 °C. Nevertheless, when the exposure time was extended to 180 and 240 min, the encapsulation improved the viability under heat.

The content of NaCl present in food is another factor that can compromise the viability of probiotics, in addition to the possibility of compromising complexes formed by proteins and polysaccharides due to the change in ionic strength. The effect of three concentrations of NaCl on the survival of lactobacilli strains is presented in Fig. 5-III. Significant differences were only observed in the results obtained in the concentration of 5% NaCl ($p < 0.05$). In this concentration, the difference between the viability values of free and encapsulated bacteria was verified ($p < 0.05$), with a reduction of 2.23 log CFU/mL and 0.41 log CFU/mL for free and encapsulated cells of *L. paracasei* BGP1, respectively, and of 2.95 log CFU/mL and 1.2 log CFU/mL for free and encapsulated cells of *L. rhamnosus* 64, respectively. Bosnea et al. (2014) encapsulated *L paraplantarum* B1 and *L. paracasei* E6 by complex coacervation and observed a protective effect of the encapsulation when the microorganisms were exposed to 3, 6, and 9% of NaCl.

As emphasized by these authors, a high salt concentration can compromise the stability of the coacervate by force of the ionic force. At the same time as microcapsules are broken, the protection of probiotics against a high concentration of NaCl is impaired. Thus, for foods that have a high salt concentration, the encapsulation technique by complex coacervation may not be the most indicated. However, it should be added that, in this study, even after the rupture of the membrane formed by the gelatin-gum Arabic complex, probiotics would still theoretically be protected from the adverse conditions of the environment, since they would be immobilized in the lipid particles.

Finally, caution is needed in order to evaluate the effect of salinity on encapsulated probiotic bacteria. Some encapsulation techniques that include unfavorable conditions to microorganisms during the process, such as heat in the spray drying technique, can promote sensitivity to salt due to the damage caused to the cytoplasmic membrane of these bacteria. Gardiner et al. (2000) reported that *L. paracasei* subsp. *paracasei* had its sensitivity to salt considerably increased after encapsulation with spray drying; the sensitivity of microorganisms to 5% NaCl was increased from 4% to 70% after the technique.

3.6. Tolerance of free or encapsulated microorganisms to simulated gastrointestinal fluids

The ability to withstand the adverse conditions of the gastrointestinal tract is an indispensable requirement for a microorganism to be classified as a probiotic of intestinal action. When the sensitivity of microorganisms to acid and bile may compromise the number of cells that will reach the intestine alive, microencapsulation technology is often used.

The sensitivity of the free strains of *L. paracasei* BGP1 and *L. rhamnosus* 64 to the simulated gastrointestinal fluids was significantly high, particularly in the gastric fluid. Free *L. rhamnosus* 64 was reduced by 1.31 log CFU/g, while free *L. paracasei* BGP1 decreased by 3.37 log CFU/g. However, the effect of the simulated intestinal fluids was small for both microorganisms. After 180 min of exposure to SIF, the population of *L. rhamnosus* 64 and *L. paracasei* BGP1 decreased by 1.64 and 1.71 log CFU/g, respectively (Fig. 6). Sensitivity reduced considerably with microencapsulation. Microencapsulated *L. paracasei* BGP1 presented a reduction of 0.66 log CFU/g in the SGF ($p > 0.05$), while *L. rhamnosus* 64 showed a reduction of 1.13 log CFU/g ($p < 0.05$). Results were also successful in the SIF, *L. paracasei* BGP1 presented a reduction of 0.75 log CFU/g, while *L. rhamnosus* 64 showed a reduction of 0.63 log CFU/g; significantly different results when comparing free and encapsulated microorganisms ($p < 0.05$). The accumulated loss (FGS + FIS) for free *L. paracasei* BGP1 was 5.08 log CFU/g, while the encapsulated form presented a loss of 1.41 log CFU/g ($p < 0.05$). The accumulated loss of free *L. rhamnosus* 64 was 2.95 log CFU/g, while the encapsulated form presented a loss of 1.76 log CFU/g ($p < 0.05$).

Microencapsulation presented satisfactory results for both the microorganisms evaluated by promoting the preservation of the viability above 6.00 log CFU/g. In a previous work by our group, in which the same technique was used to encapsulate other probiotics, the decrease of 1.2 log CFU/g (84% survival rate) after exposure to SGF and SIF was reported. These results indicate the efficiency of the encapsulation processes for protecting probiotics in the gastrointestinal tract.

3.7. Evaluation of the staibility of the encapsulated microorganisms during storage

It is known that for probiotics to achieve the desired benefits to the host's health, a threshold of microorganisms must be ingested (Ouwehand, 2017). In order to guarantee this threshold, it is necessary to ensure that microorganisms survive in the food until it is consumed. Therefore, microencapsulation should not only assure that the microorganism survives processing, but also that it remains viable during storage.

Fig. 7 shows that the microencapsulated *L. rhamnosus* 64 and *L. paracasei* BGP1 presented similar survival profiles regardless of the storage temperature. After 120 days of storage at 7 °C, there was a reduction in the concentration of viable cells of less than 1 log cycle for both microorganisms. After 120 days of storage at 25 °C, *L. rhamnosus* presented a reduction of 1.58 log CFU/g, while *L. paracasei* BGP1 had a reduction of 1.50 log CFU/g ($p < 0.05$). The comparison of the stability of the microorganisms in both storage conditions indicated that the temperature did not affect the results to the point of causing significant differences. In other words, the encapsulation technique was effective in protecting the microorganisms regardless of the storage temperature evaluated, 7 or 25 °C.

Oliveira et al. (2007a, 2007b) encapsulated *Bifidobacterium lactis* (BI01) and *Lactobacillus acidophilus* (LAC04) by means of complex

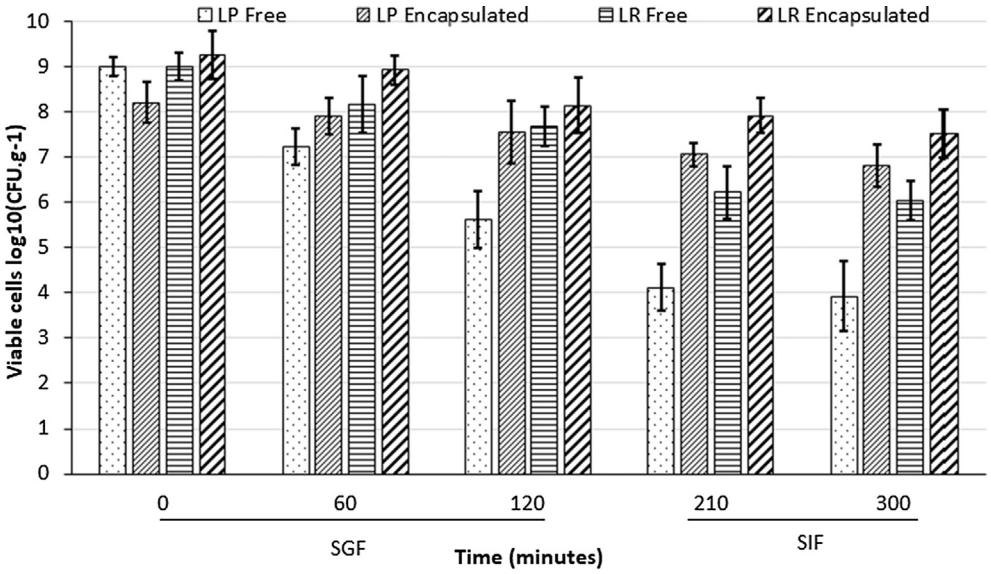

Fig. 6. Survival of free and encapsulated *L. paracasei* BGP1 (LP) and *L. rhamnosus* 64 (LR) after 300 min of exposure to simulated gastrointestinal fluids, in which 120 min were of exposure in simulated gastric fluid (SGF), and 180 min were of exposure in simulated intestinal fluids (SIF).

coacervation followed by spray drying and found that the concentration of *B. lactis* (BI 01) was reduced by 4.34 and 6.06 log cycles after the microcapsules had been stored for 120 days at 7 and 37 °C, respectively. Okuro et al. (2013) used the spray chilling technique and interesterified palm and palm kernel fat to encapsulate *L. acidophilus* with inulin or polydextrose and found that the storage temperature (−18, 7 or 20 °C) had a strong influence on the stability of the microorganisms during 120 days under controlled atmosphere conditions.

3.8. Evaluation of the preservation of the immunomodulating capacity of free and microencapsulated L. paracasei BGP1 and L. rhamnosus 64

Considering previous studies that evaluated the *in vivo* functionality of *L. paracasei* BGP1 and *L. rhamnosus* 64 strains (Gregoret, Perezlindo, Vinderola, Reinheimer, & Binetti, 2013), we assumed that these strains had the ability to stimulate the immune system, justifying their choice. However, there were no significant differences in the IgA dosage values found in the contents of the mice that received the free or the microencapsulated microorganisms ($p > 0.05$). The IgA production ranged from 56 ± 9 to 67 ± 8 pg/mL. IgA is considered the most abundant immunoglobulin in mucosal surfaces and its main function is to exert the immune exclusion of invading pathogens or foreign proteins so that its increase is considered a desirable indicator of probiotic functionality (Tabanelli et al., 2012).

Similarly, the IL-2, IL-6, IL-10 and TNF-α dosages did not present significant differences in terms of the form in which the microorganisms were administrated (free or microencapsulated) ($p > 0.05$). These results are satisfactory since they indicate that microencapsulation did not affect the immunomodulatory capacity of the strains. In addition, we verified that the microcapsule materials were well tolerated, since they did not trigger an inflammatory response, which could be evidenced by the increase of IL-2, TNF-α, and IL-6 cytokines.

Recently, Zacarías et al. (2017) evaluated the effect of spray drying or lyophilization on the preservation of the functionality of *Bifidobacterium lactis* INL1 isolated from breast milk. Using the dosage of IgA, IL-10, and IFN-γ, the authors found that the process affected the immunological stimulation capacity of the strains. The most striking differences in results were found between the mice that received the free and the lyophilized strains. In the present study, the microcapsules containing both strains of lactobacilli were subjected to lyophilization drying, however, in contrast to what was reported by Zacarías et al. (2017), this process did not compromise the *in vivo* immunomodulatory capacity, which may be considered a very positive result.

Lavari et al. (2017) also used *L. rhamnosus* 64 and found that its ability to immunomodulate healthy animals was modified, particularly the cytokine profile, when the microorganism was spray dried in a solution of whey and starch. Despite the use of the same strain, the results obtained by Lavari et al. (2017) concerning the cytokine dosages were considerably higher than those found in the present study. One possible explanation for this difference could be related to the dosage administered to mice. While Lavari et al. (2017) administered 10^8 CFU/ml, only 10^7 CFU/ml was administered in the present study due to the viscosity of the suspension containing the microparticles loaded with *L. paracasei* BGP1 or *L. rhamnosus* 64, which made it difficult to perform the gavage technique. Although the microcapsules produced had 10^8 CFU/g, a 1:10 dilution was necessary, resulting in an administration of approximately 1×10^7 CFU/animal. Studies have shown that the dosage received has a significant influence on the induction of the immune response (Gill & Rutherfurd, 2001; Ya et al., 2008).

Still considering the effect of technological processing, Tabanelli et al. (2012) studied the effect of homogenization under high pressure on the *in vivo* functionality of *L. paracasei* A13 and found that cells subjected to this processing induced a higher IgA response compared to cells that were not exposed to homogenization under high pressure. The authors stated that this superior performance in terms of the immunological stimulation of these bacteria could be related to the increased hydrophobicity of the cell wall. Cellular hydrophobicity is considered an important factor for the interaction of probiotic bacteria with the gut and the associated immune cells (Burns, Reinheimer, & Vinderola, 2011). Furthermore, the hydrophobicity of the cell wall has been related to strain adherence (Basson, Flemming, & Chenia, 2008).

In 2011, Bajracharya et al. evaluated the effect of microencapsulation of *L. salivarus* 29 in alginate/chitosan/alginate microcapsules on the induction of cytokines *in vitro* and found that the free bacteria had a greater ability to stimulate TNF-α and IL-10 when compared to the microencapsulated bacteria. While the authors did not present possible explanations for this result, they believed in a relation between the controlled release and the immunostimulation of the macrophage cells. The exposure time of the encapsulated bacteria would be reduced in comparison to the free bacteria as a function of the time required for the release of the microorganisms from the capsule. Similar results were reported by Jiang et al. (2013) when using alginate/chitosan/alginate to encapsulate *L. plantarum* 25.

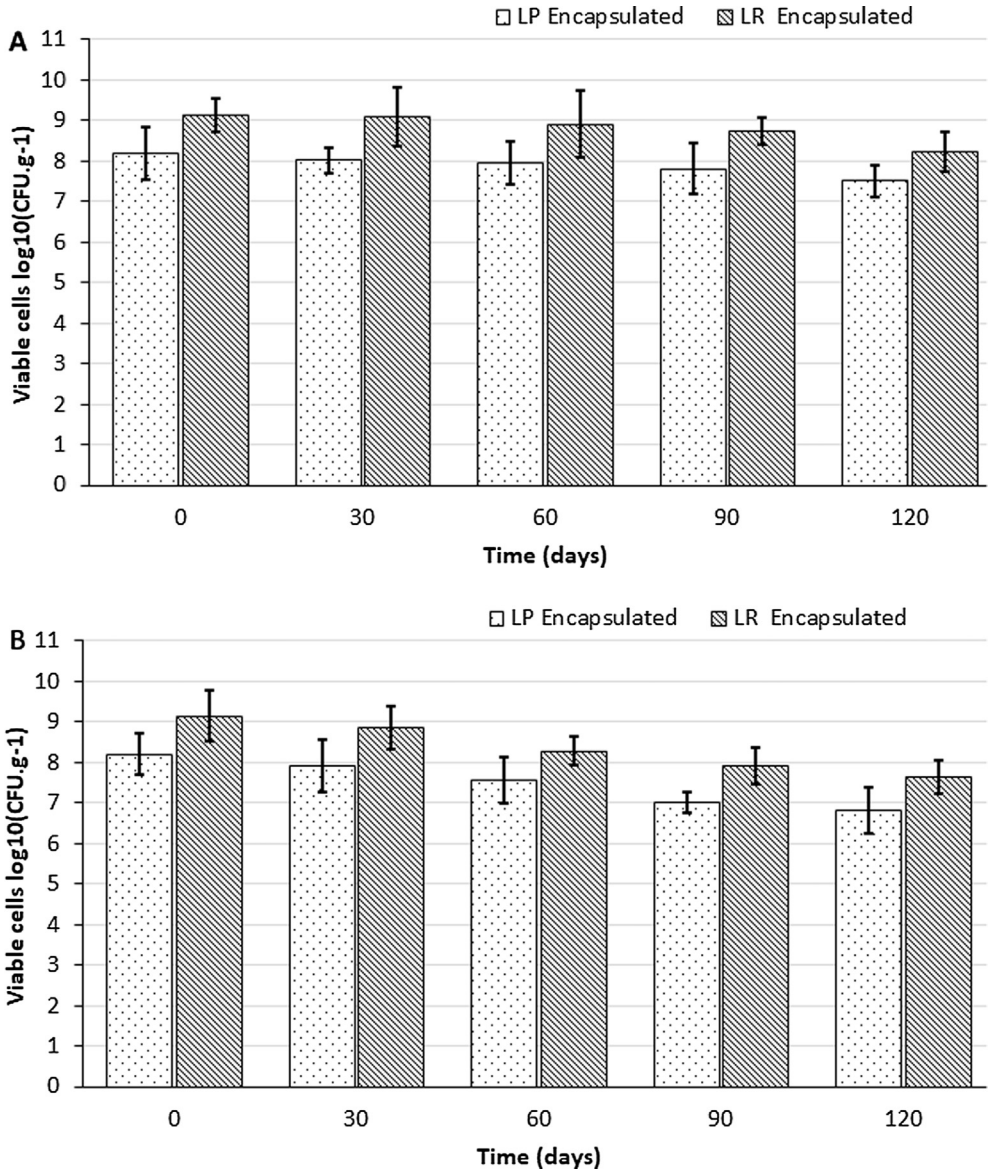

Fig. 7. Viability of *L. paracasei* BGP1(LP) and *L. rhamnosus* 64 (LR) encapsulated during storage at 7 °C (A) and 25 °C (B) during 120 days.

4. Conclusions

The immobilization of *L. paracasei* BGP1 and *L. rhamnosus* 64 in lipid particles coated by electrostatic interaction of gelatin and gum Arabic provides protection under adverse conditions found in food processing, storage, and passage through the gastrointestinal tract. The hydrophobicity of the probiotic cell wall positively influences the encapsulation efficiency considering the suggested method.

The microencapsulation process performed in this study does not compromise the immunostimulatory capacity of the probiotics. Thus, the results obtained support the use of this technique in new studies that aim to apply both strains in food matrices, as well as to reinforce the microcapsule wall in order to provide greater thermal stability to the probiotics.

Ethics statements

The project was approved by the Ethics and Research Committee of the Universidad Nacional del Litoral prior to the beginning of the study.

Conflict of interest

None.

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