Layer-by-layer encapsulation of *Lactobacillus delbrueckii* subsp. *Bulgaricus* using block-copolymers of poly(acrylic acid) and pluronic for safe release in gastro-intestinal conditions

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Keywords: Lactic acid bacteria, Polyacrylic acid, Lactic acid bacteria, Pluronic, Pluronic, Storage, Bacterial safe delivery

1. Introduction

Lactic acid bacteria have an important role in the food and pharmaceutical industries, as they are extensively used as starters in the development of food and probiotic products. To exert their action, adequate concentrations of viable bacteria must arrive to the gut. However, before reaching the gut, microorganisms must overcome different challenges. As lactic acid bacteria are mainly orally administered, they are exposed to different harmful environments, namely the low pH of the stomach, bile salts and enzymatic activities. To overcome these defies adequate strategies are required. Bacterial microencapsulation into polymer matrices may protect them from the gastro-intestinal environment, also allowing bacterial release to the target site (the gut) and ensuring a mechanical integrity during both the production process and storage. Therefore, an adequate selection of the coating materials and of the microencapsulation technique is mandatory (Priya, Vijayalakshmi, & Raichur, 2011; Zheng et al., 2017).

Among the large number of encapsulation methods, the layer-by-layer self-assembly technique (LbL) is a unique and simple procedure based on the surface charge of the material or compound to be retained. It consists in the alternative deposition of oppositely charged polyelectrolytes to form nanostructured functional thin films whose properties can be precisely controlled by manipulating the pH and the concentrations of the polyelectrolytes (Decher, Hong, & Schmitt, 1992; Kahraman, Zamaleeva, Fakhrrullin, & Culha, 2009). This strategy enables a complete encapsulation of...
the material whose thickness can be controlled at a nanometric scale. Furthermore, LbL allows the passage of small and large molecules and can be used to encapsulate both living and no living particles with different surface charges, shapes and sizes (Yucel Falco, Sotres, Rascon, Risbo, & Cardenas, 2017). This versatility makes it an interesting solution to microencapsulate sensitive lactic acid bacteria. Other works reported the efficiency of LbL to encapsulate certain lactobacilli species using chitosan, alginate or carbonyl methylcellullose as polyelectrolytes (Cook, Tzortzis, Khutoryanskiy, & Charalampopoulos, 2013; Decher et al., 1992; Kahraman et al., 2009; Priya et al., 2011; Tang, Wang, Podsadiolo, & Kotov, 2006; Yucel Falco et al., 2017). Chitosan (CHI) is a remarkable biopolymer due to its organic nature, resistance, degradability and capacity to be converted into a cationic polyelectrolyte (pKa: 6.5) (Coimbra et al., 2011; Rinaudo, 2006). This way, as the bacterial surface is negatively charged, CHI is generally used as first positive layer. As negative layers, alginate and carboxymethylcellulose have been mainly used hereto (Cook et al., 2013; Decher et al., 1992; Khutoryanskiy, & Charalampopoulos, 2013; Decher et al., 1992; Priya et al., 2011; Tang et al., 2006; Yucel Falco et al., 2017).

Using pH-sensitive polyelectrolytes is a recent and promising approach to deliver different kinds of drugs (Guzmán et al., 2011). In this regard, some monomers as acrylic or methacrylic acid can be used to obtain pH-sensitive polymer complexes (Alves et al., 2013). Poly(acrylic acid) (PAA) is a biocompatible and biodegradable anionic polyelectrolyte (pKa: 4.95), abundantly used in pharmaceutical and cosmetic formulations (Seiki & Tirrell, 1984), that precipitates at low pH and swells and dissolves at high pH. The use of CHI and PAA as polyelectrolytes of opposite charges by the LbL technique was already reported in literature (Guzmán et al., 2011), and revealed to be a permeable high performance promise drug and storage delivery system.

Pluronic™ (PLU) is composed of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a basic and hydrophobic EO–PO–EO structure. PLU exists as a commercial product in a wide range of molecular weights and architectures, determining its hydrophilic and lipophilic properties (Barreiro-Iglesias et al., 2005; Kabanov, Batrakova, & Alakhov, 2002). pH-sensitive block-copolymers of PAA and PLU (PAA-PLU-PAA or PPP) can be obtained using the reactive terminal OH groups of the PLU chain to start a living radical polymerization by emulsion for the growth of the PAA chains (Alakhov et al., 2004; Chandaroy, Sen, & Hui, 2001; Choo, Yu, & Xue, 2011; Ma, Xu, Nie, & Pan, 2008). The hydrophobic properties of PLU and the pH responsive behavior of PAA have converted PPP copolymers in a pH-sensitive block-copolymer of the mirror type with multipurpose excipients able to enhance the aqueous solubility and stability of drugs. For this reason, they have been used for the controlled delivery of drugs to the gut (Barreiro-Iglesias et al., 2005; Kabanov et al., 2002). Despite their wide employment in the pharmaceutical industry, their use as lactic acid bacteria coating has never been reported hitherto. Considering that lactic acid bacteria must overcome the harmful gastro-intestinal environment to be safely released in the gut, using PPP as a coating layer in LbL technique appears as an interesting strategy to preserve bacterial integrity during the passage through the gastro-intestinal tract.

The aim of this work was to use LbL technique to encapsulate Lactobacillus delbrueckii subsp. bulgaricus CIDCA 333, a particularly sensitive strain to any kind of stress (Romano, Schebor, Mobili, & Gomez-Zavaglia, 2016; Tymczyszyn et al., 2008, 2012; Tymczyszyn, Gerbino, Illanes, & Gomez-Zavaglia, 2011). CHI was used as cationic polymer and two different molecular weight PLU were used to synthesize the anionic PPP polyelectrolyte (Pluronic F127, with 12,600 Da, F127) and Pluronic F68, with 8400 Da, F68). Microorganisms were encapsulated using different numbers of layers. They were plate counted before and after coating, after freeze-drying, and after exposure to simulated gastric and intestinal conditions. The efficiency of encapsulation was determined using confocal microscopy. Then, the toxicity of the coated bacteria using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were assessed. To complete the investigation, bacterial stability was determined during storage at 4 °C for 60 days. The results obtained revealed the promising potential of this PPP as delivery system for lactic acid bacteria in food products.

2. Materials and methods

2.1. Materials

Chitosan (MW 100,000 to 300,000, Acros Organics, NJ, USA), Acrylic Acid (Sigma-Aldrich, St. Louis, MO, USA), Pluronic F68 (Sigma-Aldrich, St. Louis, MO, USA), Pluronic F127 (BASF Chemicals, NJ, USA), Tween 80 (Sigma-Aldrich, St. Louis, MO, USA), lauroyl peroxide (Sigma-Aldrich, St. Louis, MO, USA), 4,4’-azobis-(4-cyanovaleric acid) (Sigma-Aldrich, St. Louis, MO, USA), hexane (VWR Chemicals, MA, USA), sodium chloride (Sigma-Aldrich, St. Louis, MO, USA), sodium hydroxide (Panreac, Madrid, Spain), hydrochloric acid (Fisher Scientific, USA), potassium chloride (Anendra, Buenos Aires, Argentina), sodium bicarbonate (Biopack, Buenos Aires, Argentina), sodium hydrogen phosphate (Anendra, Buenos Aires, Argentina), potassium dihydrogen phosphate (J.T. Baker, NJ, USA), MRS broth (Biokar Diagnostics, France), agar (Parafarm, Buenos Aires, Argentina), sucrose (Biopack, Buenos Aires, Argentina), peptone (P1625, Sigma-Aldrich, St. Louis, MO, USA), pepsin (P7125, Sigma-Aldrich, St. Louis, MO, USA) and bile salts (P48305, Sigma-Aldrich, St. Louis, MO, USA), FITC-inulin (F3272, Sigma-Aldrich, St. Louis, MO, USA), To-Pro™-3 iodide (Thermofisher Scientific, NJ, USA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Methods

2.2.1. Block-copolymer synthesis

2.2.1.1. Poly(acrylic acid)-Pluronic-Poly(acrylic acid) (PAA-PLU-PAA or PPP). The synthesis of PPP was performed through polymerization by emulsion (Alakhov et al., 2004; Choo et al., 2011; Ma et al., 2008). Briefly, Pluronic™ (PLU) (F127 or F68), with different molecular weight, was dissolved in partially neutralized acrylic acid (AA) by addition of 0.5 ml NaOH 5 M solution under constant agitation and nitrogen flow in another seal flask. AA was proportionally added considering the number of —OH terminal groups present in each PLU to obtain two copolymers with equal AA repeating units. 100 mg lauroyl peroxide and 100 mg 4-azobis-(4-cyanoveralic acid) were dissolved in 5 ml AA, and then added to the PLU solution. This final solution was introduced into a 500 mL round bottom 3-necked flask with 250 mL Tween 80 solution in nonane (1% v/v) previously deoxygenated for 1 h through a constant nitrogen flow. The reactor was heated and maintained at 70 °C for 10 h. The obtained polymer, a white rubbery agglomerate, was washed in hexane and water to remove impurities, and dried in a vacuum oven at 40 °C for 48 h. The schematic reaction of this procedure is presented in Fig. 1.

2.2.2. Block-copolymer characterization

2.2.2.1. Nuclear Magnetic Resonance (NMR). 1H NMR spectra were obtained using a Bruker Advance III 400 MHz spectrometer 9.4 T (Karlsruhe, Germany). Samples were dissolved in d6-THF, and analyzed in tubes of 5 mm diameter, using tetramethylsilane as internal standard.
2.2.2.2. Attenuated total reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR). ATR-FTIR spectra were recorded on a Thermo Nicolet iS10 spectrometer (Thermo Scientific, NJ, USA) equipped with a Golden Gate Single Reflection Diamond ATR accessory. Spectra were obtained in the 4000–400 cm⁻¹ range, by co-adding 64 scans with 4 cm⁻¹ spectral resolution.

2.2.3. Bacterial strains and growth conditions
Lactobacillus delbrueckii subsp. bulgaricus CIDCA 333 was isolated from fermented milk (Gomez-Zavaglia, Abraham, Giorgieri, & De Antoni, 1999) and maintained frozen at −80 °C in 120 g/L non-fat milk solids. Microorganisms were cultured twice in MRS broth (de Man, Rogosa, & Sharpe, 1960) at 37 °C in aerobic conditions. Cultures in the stationary phase (~5 × 10⁸ CFU/mL) (Tymczyszyn et al., 2011) were harvested by centrifugation (Hermle Centrifuge Z 326 K, Gosheim, Germany) at 6000 rpm for 10 min and washed twice with 0.15 M NaCl. The pellets were used for layer-by-layer encapsulation.

2.2.4. Layer-by-layer encapsulation (LbL)
CHI and PPP were dissolved in 0.15 M NaCl at pH 6 to obtain 0.005 mM and 0.04 mM solutions, respectively. Since microorganisms are negatively charged the first layer to be deposited was the positively charged polyelectrolyte (CHI). Taking this into account, the bacterial pellets obtained in Section 2.2.3 were suspended in 20 mL CHI solution to attain 10⁷–10⁸ CFU/mL. The deposition of each layer was evaluated at 37 °C by monitoring the zeta potential on a Malvern Instrument Zetasizer Nano-Z (Malvern Instruments, Malvern, UK), using a combination of measurement techniques: electrophoresis and laser doppler velocimetry (Laser Doppler Electrophoresis). Cells were suspended in milli-Q water, and the zeta potential was provided directly by the instrument.

After the addition of each polymer layer, coated bacteria were inoculated in MRS broth (2% v/v) and incubated at 37 °C. Growth kinetics were followed by determining the absorbance at 600 nm every 60 min. Non-coated bacteria were used as controls.

2.2.5. Confocal laser scanning microscopy analysis
The size, integrity, and the permeability of the coated and uncoated microorganisms were determined using a confocal laser-scanning microscope (Leica TCS SP5 Leica Microsystems, Wetzlar, Germany). Microorganisms were visualized using two dyes: macromolecular fluorescein isothiocyanate (FITC) bound to inulin, and To-Pro⁺-3 iodide, which is a DNA staining dye. Fresh samples of coated or uncoated cells were suspended in distilled deionized water at a concentration of 10⁸ cells/mL. First FITC-inulin (final concentration: 0.8 μM) was added and allowed to interact with the bacteria for 60 min at 37 °C. Then, samples were fixed by adding methanol (25 % v/v) for 5 min at 20 °C. Finally, cells were stained with To-Pro⁺-3 iodide (final concentration: 1 μM) for 5 min at 37 °C.

An aliquot of the suspension was dispersed on a glass slide and dried under a flow of sterile air. The excitation wavelength was 488 nm and 633 for FITC-inulin and To-Pro⁺-3 iodide, respectively.

2.2.6. Freeze-drying and storage
For freeze-drying assays, the coated bacteria were suspended in a 20 % w/v sucrose solution. Suspensions were frozen at −80 °C for 12 h, and freeze-dried (Rificor L-A-B4-C, Buenos Aires, Argentina), operating with the condenser at −45 °C in a chamber pressure of 0.04 mbar. The freeze-drying process lasted for 48 h. Non-coated microorganisms suspended in 20 % w/v sucrose were used as controls.
Freeze-dried-coated and uncoated microorganisms were serially diluted in 0.15 M NaCl, plated on MRS agar and incubated at 37°C in aerobic conditions for 48 h. Plate counts were expressed as log CFU/mL before and after the process. Freeze-dried microorganisms were stored at 4°C and plate counted at regular intervals during 60 days.

2.2.7. Simulated gastric and intestinal fluid

Experiments were carried out on the freeze-dried samples. The assay was divided into two stages: gastric digestion (pepsin, pH 2.5) and intestinal digestion (pancreatin, bile salts, pH 8) (adapted from Grimoud et al., 2010). In brief, coated and uncoated microorganisms were suspended in simulated gastric solution (3200 units/mL porcine pepsin, 7.2 mM CaCl2, 98 mM NaCl, 13.6 mM KCl, 0.82 mM KH2PO4, pH 2.5) and incubated for 90 min at 37°C under continuous shaking (100 rpm, MaxQ 4000, Thermo Scientific, USA). Afterwards, bacteria were centrifuged at 6000 rpm for 5 min, and washed once with phosphate buffer saline (PBS) (0.82 mM K2HPO4; 98 mM NaCl; 5.5 mM Na2HPO4). Finally pellets were suspended in simulated intestinal fluid (0.1% w/v pancreatin, 0.15% w/v bovine bile salts, 22 mM NaCl, 3.2 mM KCl, 7.6 mM NaHCO3, pH: 8). The resulting solution was incubated for 90 min at 37°C under continuous shaking at 100 rpm (MaxQ 4000, Thermo Scientific, NJ, USA).

After each digestion step, aliquots of 1 mL of uncoated and coated bacteria were diluted in 0.15 M NaCl, plate counted on MRS agar and incubated at 37°C for 48 h in aerobic conditions. The results were expressed in log CFU/mL.

2.2.8. Mitochondrial dehydrogenase activity

The potential toxicity of polymers on intestinal cells was determined by assessing mitochondrial dehydrogenase activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Caco-2/TC7 cells were seeded in duplicate in 24-well plates at 1 x 105 cells per well and incubated for 5 days. Afterwards, coated and uncoated microorganisms were added at a concentration of 1 x 106 CFU/mL and cells were incubated for 24 h. Then, cells were washed twice with PBS (pH 7.0), and the medium was replaced by Dulbecco’s Modified Eagle’s Medium (DMEM) (without phenol red dye) containing 0.5% w/v insulin, 0.15% w/v bovine serum albumin, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The ATR-FTIR spectra of PLU, AA, PPP12 and PPP24 showed clear differences among the four compounds (Fig. 2B). In the 3600–3400 cm−1 region, a wide band corresponding to the νOH vibrational mode of the carboxylic acid side-group was observed in the AA, PPP12 and PPP24 spectra. The broader bands in the 3000–2800 cm−1 region can be assigned to the νCH vibrational modes of all the four compounds investigated. The νC=O vibration was observed at 1713 cm−1 in PPP24 and at 1702 cm−1 in PPP12. As carboxyls involved in hydrogen bonds absorb at lower wavenumbers, the noticeable shift of the νC=O band of PPP12 to lower wavenumbers indicates a stronger hydrogen-bonds network in this polymer. The group of bands at ~1400 cm−1 can be ascribed to the νCH vibrational mode, present in all the four compounds. The band at 1095 cm−1 corresponds to the νOC of PLU, also observed in PPP12 and PPP24 (Fig. 2B).

Fig. 3 shows the evolution of superficial charges after adding each polymer layer. The negatively charged bacterial surface acted as the substrate for the adsorption of CHI as the first layer. The alternate more and less negative values of zeta potential can be considered as a proof of a correct self-assembly of polymers on the bacterial surface.

To evaluate the effect of coating on bacterial growth, growth kinetics were carried out after adding each layer and compared with those of uncoated bacteria (Fig. 4). They were adjusted to Eq. (2):

\[
Abs(t) = \frac{Abs_0 - Abs_f}{1 + e^{-c(t-p)}} + Abs_f
\]

where \( t \) is the time in hours, \( Abs_0 \) is the absorbance at 600 nm at time equal to 0, \( Abs_f \) is the absorbance at 600 nm once attained the stationary phase, \( c \) is the time corresponding to the inflection point and \( p \) is an exponential fitting factor. The lag time was calculated as the intersection between the tangent line at \( t = c \) and \( Abs_0 \). The growing rate during the exponential phase was calculated as the module of the slope of the tangent line. The values of the lag time and growth rate for all the growth kinetics are shown in Table 1. The addition of CHI and PPP increased the lag time regardless the number of layers covering bacteria. Three different kinetic

3. Results

To evaluate the influence of the molecular weight on bacterial encapsulation, two different molecular weight PLU were used: Pluronic F127 (with 12,600 Da, F127) and Pluronic F68 (with 8400 Da, F68). Taking this into account, AA was proportionally added to obtain two copolymers with equal AA repeating units. This resulted in two synthesized PPP with different molecular weights. The scheme of the chemical reaction is shown in Fig. 1.

The chemical structure of both obtained copolymers, together with that of the corresponding reagents (PLU and AA) was assessed by ATR-FTIR and 1H NMR (Fig. 2). As both the ATR-FTIR and 1H NMR spectra of PLU F68 and F127 were similar, only one of them is shown. Fig. 2A (1) shows the 1H NMR spectrum of PLU, where the peak at 1.1 ppm (zoomed in the figure and indicated as a) is assigned to the methyl hydrogen of PLU segment (−CH−CH3), and at 1.6 ppm the terminal −OH group can be observed. In the spectra of AA (Fig. 2A (2)) it is evident the characteristic vinylic group (HC−CH2) chemical shift at 5.5–6.5 ppm (peaks c, d and e in the figure). These later peaks completely disappeared in the PPP (Fig. 2A (3 and 4)) spectra, indicating that the obtained copolymers were successfully purified with no residual AA monomer present. The characteristic methine hydrogen (−CH−CH2) of the AA units at 2.2–2.8 ppm (peak g in the figure) and the methyl hydrogen of PLU segment (−CH−CH3) at 1.1 ppm (peak a in Fig. 2A (3) and (4)) were also evident. These features prove the success of the PPP synthesis.

From the integration of the PLU and AA characteristic peaks, at 1.1 ppm and 2.2–2.8 ppm, respectively, it was possible to estimate the molecular weight of the obtained polymers. Considering the molecular weights of both PPP copolymers shown in Fig. 1, from now on they will be named as PPP12 (~12 kDa) and PPP24 (~24 kDa).

The ATR-FTIR spectra of PLU, AA, PPP12 and PPP24 showed clear differences among the four compounds (Fig. 2B). In the 3600–3400 cm−1 region, a wide band corresponding to the νOH vibrational mode of the carboxylic acid side-group was observed in the AA, PPP12 and PPP24 spectra. The narrower bands in the 3000–2800 cm−1 region can be assigned to the νCH vibrational modes of all the four compounds investigated. The νC=O vibrational mode was observed at 1713 cm−1 in PPP24 and at 1702 cm−1 in PPP12. As carboxyls involved in hydrogen bonds absorb at lower wavenumbers, the noticeable shift of the νC=O band of PPP12 to lower wavenumbers indicates a stronger hydrogen-bonds network in this polymer. The group of bands at ~1400 cm−1 can be ascribed to the νCH vibrational mode, present in all the four compounds. The band at 1095 cm−1 corresponds to the νOC of PLU, also observed in PPP12 and PPP24 (Fig. 2B).

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\]

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Fig. 2. (A) $^1$H NMR spectra of PLU (1), AA (2), PPP12 (3) and PPP24 (4) in THF-d$_8$ at 400 MHz. (B) ATR-FTIR spectra of PLU, AA, PPP12 and PPP24.
profiles were observed: after adding the first CHI layer [(CHI/PPP) 1/0], the lag time increased 2.8 h with regard to that of uncoated bacteria. Bacteria coated with [(CHI/PPP)1/1] and [(CHI/PPP)2/1] showed similar patterns, the lag time being 12.0 h when using PPP24 and 14.3 or 14.8 when using PPP24. The third profile was that of bacteria coated with [(CHI/PPP2/2), which showed the largest lag times both when using PPP12 and PPP24 (Fig. 4 and Table 1). It is interesting to note that the larger the lag time, the higher the growth rate. In particular, the growth rate of bacteria coated with [(CHI/PPP2/2)] was about twice that of uncoated bacteria (controls) (Table 1).

Fig. 5 shows L. delbrueckii subsp. bulgaricus CIDCA 333 cultivability after adding each polyelectrolyte layer and further freeze-drying. Coated microorganisms freeze-dried in the absence of sucrose did not overcome the freeze-drying process (data not shown). For this reason, freeze-drying was carried out in the presence of sucrose. Before freeze-drying, the cultivability of microorganisms coated with up to 4 layers of CHI and PPP12 (or PPP24) was not significantly different from that of uncoated fresh cultures (p < 0.05) (white bars in Fig. 5). After freeze-drying, the cultivability of bacteria coated with [(CHI/PPP12)2/0], [(CHI/PPP12)1/1] and [(CHI/PPP12)2/2] was not significantly different from that of the corresponding coated bacteria before freeze-drying (p > 0.05). When adding the second PPP12 layer [(CHI/PPP12)2/2], the cultivability significantly dropped up to 6.28 ± 0.06 logarithmic units (p < 0.05). In turn, freeze-dried bacteria coated with CHI/PPP24 showed a significant decrease of cultivability already after adding the first PPP24 layer [(CHI/PPP24)1/1] (p < 0.05) (Fig. 5B).

The permeability of bacterial membranes after adding the different polymer layers was investigated by confocal microscopy using FITC-inulin (high molecular weight polymer) and To-Pro®-3 iodide (low molecular weight DNA stain). Fig. 6 shows the penetration of these two fluorescent dyes. Uncoated cells incorporated both To-Pro®-3 iodide and FITC-inulin easily. The concomitant observation of green and red fluorescence (corresponding to FITC-inulin and To-Pro®-3 iodide, respectively) inside the cells indicates that cell membranes are permeable to both low and high molecular weight molecules (Fig. 6A). In turn, the addition of one or more layers of CHI and PPP hindered the internalization of the higher molecular weight FITC-inulin dye, and only the smallest one (To-Pro®-3 iodide) could cross the membranes (Fig. 6B and C). In this latter case, confocal images showed that the green fluorescence remained outside the cells whereas red fluorescence was inside them. In addition, merged images of bacteria coated with [(CHI/PPP12)2/1] showed a higher intensity of the red stain in regard with bacteria coated with [(CHI/PPP12)2/2] (Fig. 6B and C).

After the exposure to simulated gastric and intestinal conditions, the cultivability of uncoated freeze-dried L. delbrueckii subsp. bulgaricus CIDCA 333 significantly decreased from 8.35 ± 0.10 to 4.23 ± 0.07 and 4.95 ± 0.01 log CFU/mL, respectively (Fig. 7). A similar behavior was observed for bacteria coated only with CHI [(CHI/PPP)1/0]. The addition of PPP12 and PPP24 protected microorganisms from the harmful environment of the gastro-intestinal tract.

<table>
<thead>
<tr>
<th>Coating</th>
<th>lag time (h)</th>
<th>Growth rate (absorbance units/h)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-coated</td>
<td>8.4</td>
<td>0.18</td>
<td>0.995</td>
</tr>
<tr>
<td>CHI/PPP12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHI/PPP1/0</td>
<td>11.2</td>
<td>0.17</td>
<td>0.999</td>
</tr>
<tr>
<td>CHI/PPP1/1</td>
<td>12.0</td>
<td>0.21</td>
<td>0.996</td>
</tr>
<tr>
<td>CHI/PPP2/1</td>
<td>12.0</td>
<td>0.20</td>
<td>0.996</td>
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<tr>
<td>CHI/PPP2/2</td>
<td>19.5</td>
<td>0.38</td>
<td>0.997</td>
</tr>
<tr>
<td>CHI/PPP24</td>
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<tr>
<td>CHI/PPP1/0</td>
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<tr>
<td>CHI/PPP2/2</td>
<td>19.5</td>
<td>0.32</td>
<td>0.995</td>
</tr>
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In this sense, the cultivability of freeze-dried microorganisms coated with [(CHI/PPP)1/1], [(CHI/PPP)2/1] and [(CHI/PPP)2/2] exposed to simulated gastric and intestinal conditions did not significantly decrease regarding that of the corresponding coated microorganisms before exposure. This behavior was observed for both CHI/PPP12 and CHI/PPP24 (Fig. 7). Taking this into account, further studies were carried out with bacteria coated with [(CHI/PPP)1/1] and [(CHI/PPP)2/1] (Figs. 8 and S1). These conditions are easier to obtain than [(CHI/PPP)2/2] and at the same time guarantee the gastro-intestinal protection of microorganisms.

MTT assays revealed that neither CHI nor PPP were cytotoxic for the Caco-2/TC7 cell line. Indeed, monolayers incubated for 24 h with coated bacteria exhibited no significant decays of viability for both PPP12 and PPP24 (Fig. S1).

Storage of coated microorganisms at 4 °C showed no significant decrease of cultivability up to 60 days of storage (Fig. S2).

4. Discussion

The LbL technique enables a complete and controlled encapsulation, by regulating the number of polyelectrolyte layers at a nanometric level. Although this technique dates back from several years and is very simple to be implemented, it has been scarcely used for encapsulation of lactic acid bacteria (Priya et al., 2011; Yucel Falco et al., 2017). In this work, block-copolymers composed of PLU and PAA (e.g.: PPP12 and PPP24) were used to microencapsulate L. delbrueckii subsp. bulgaricus CIDCA 333, a strain extremely sensitive to any kind of stress, including preservation processes and gastro-intestinal environment (Romano et al., 2016; Tymczyszyn et al., 2008, 2011, 2012). PPP is a pH-sensitive copolymer that has demonstrated to be efficient for the controlled delivery of drugs in the gut (Barreiro-Iglesias et al., 2005; Kabanov et al., 2002) but, to the best of our knowledge, had never been used to encapsulate lactic acid bacteria. For this reason, the use of LbL as encapsulation method and PPP as coating material showed to be a promising strategy to microencapsulate L. delbrueckii subsp. bulgaricus CIDCA 333, and ensure the safe arrival to the gut. In addition, combining PPP with CHI in a self-assemble LbL encapsulation technique resulted in an adequate controlled release system.

Fitting the experimental information of the growth kinetics obtained after adding each polyelectrolyte layer led to interesting information (Fig. 3 and Table 1). The lag time of coated microorganisms was larger than that of uncoated ones. This observation can be ascribed to the fact that polyelectrolyte layers must be firstly hydrated to allow bacterial liberation and access to the nutrients present in the growth medium. This also explains that the higher the number of layers the larger the lag time.

Coated microorganisms freeze-dried without sucrose did not overcome the freeze-drying process (data not shown). As the goal of this work was to evaluate the role of PPP on LbL bacteria encapsulation, we decided to use sucrose as lyoprotectant, a well-known protective compound (Tymczyszyn, Díaz, Gómez-Zavaglia, & Disalvo, 2007), and rule out any detrimental effect ascribable to the intrinsic sensitivity of the strain to the freeze-drying process. An integrative analysis of the growth kinetics, the cultivability before and after freeze-drying and after the exposure to gastric and intestinal conditions (Figs. 3, 5 and 7), allowed us to elucidate the role of each polyelectrolyte layer on the encapsulation process. Although the addition of a layer of CHI [(CHI/PPP)1/0] was the condition in which the lag time increased the least and no significant differences were observed after freeze-drying (Fig. 5), bacteria freeze-dried just with CHI were more prone to gastro-intestinal conditions (Fig. 7). On the other extreme, bacteria coated with two layers of each polymer [(CHI/PPP)2/2] had the largest lag times (Fig. 3 and Table 1) but the highest growth rate (Table 1). This indicates that hydration of four polyelectrolyte layers required more time (lag time 19.5 h), but once microorganisms were released, they grew faster (0.32–0.38 absorbance units/hour, Table 1). However, microorganisms coated in these conditions were more susceptible to the freeze-drying process (Fig. 5). Bacteria coated with two or three layers, [(CHI/PPP)1/1] and [(CHI/PPP)2/1], behaved similarly regarding lag time and growth rates (Table 1). However, the effect of PPP12 and PPP24 was different. Microorganisms coated with PPP12 were better protected during freeze-drying than those coated with PPP24 (Fig. 5). Hence, one can think that the stronger nature of the hydrogen-bonds network in PPP12 (Fig. 2B), leading to a more resistant material, could be related with the higher resistance of PPP12 coated-bacteria to the freeze-drying process (Fig. 5A).

The efficiency of both PPP12 and PPP24 as control release systems was clearly demonstrated by analyzing the cultivability after exposure to gastro-intestinal conditions (Fig. 7). In fact, under this circumstance the cultivability of bacteria coated with [(CHI/PPP)1/1] and [(CHI/PPP)2/1] was significantly higher than that of uncoated bacteria or bacteria coated with a single layer of CHI [(CHI/PPP)1/0] (Fig. 7). Bacteria coated with [(CHI/PPP)2/2] behaved in a similar way, but as the increase of the number of layers delayed the growth kinetics (Fig. 4), [(CHI/PPP)1/1] and [(CHI/PPP)2/1] appeared as a sufficient number of layers to protect microorganisms from gastric and intestinal conditions. Due to the PPP resistance to acidic medium, only a slight decrease of

Fig. 5. Log CFU/mL of uncoated and coated L. delbrueckii subsp. bulgaricus CIDCA 333 before and after freeze-drying in the presence of 20 % w/v sucrose. Slashed bars denote cultivability before the process and white bars, after the process. Statistical analysis was carried out by two-way ANOVA, and statistical significance was set at p < 0.05. Different letters indicate statistically significant differences. (A) Bacteria coated with CHI and PPP12 layers. (B) Bacteria coated with CHI and PPP24 layers.
cultivability was observed after the exposure to gastric conditions (Fig. 7A and B). Thus, the capsules may not release their content in the acidic medium of the gastric environment. Once attained the intestinal conditions, and owing to the solubility of PPP in basic media, the capsules released their bacterial content and cultivability increased (Fig. 7).

The analysis of confocal images clearly indicated that the addition of one or more polyelectrolyte layer, (CHI or PPP) excluded or delayed the internalization of high molecular weight compounds as FITC-inulin (Fig. 6). However, coated or uncoated cells showed a similar incorporation of small compounds as To-Pro-3 iodide stain, indicating an adequate permeability for molecules of that size. It is important to point out that, coated cells were capable to incorporate To-Pro-3 iodide but the incorporation of a further layer, as for [(CHI/PPP12)2/1] bacteria, slightly decreased its penetration. This confirms the existence of an extra barrier to be overcome by the fluorescent stain when extra layers are deposited. To-Pro-3 iodide has a molecular weight of 671.42. Hence, coated bacteria exhibited a good permeability for molecules such as monosaccharides, disaccharides, amino acids and di or tripeptides, which constitute the main source of carbon and energy for bacterial growth. In addition, encapsulation could act as a barrier to prevent the contact or internalization of bacteriophages, bacteriocins or potential harmful enzymes.

Finally, combining sucrose as lyoprotectant and PPP for encapsulation led to significant results regarding storage (Fig. S2). The observation of no significant decrease of cultivability after 60 days of storage underlines the relevance of the proposed strategy to pro-

Fig. 6. Confocal laser scanning micrographs of uncoated (A) and coated (B, C) L. delbrueckii subsp. bulgaricus CIDCA 333. Coated cells correspond to [(CHI/PPP12)1/1] (B) and [(CHI/PPP12)2/1] (C) cells. Green fluorescence is shown on the left panel, red fluorescence is shown in the middle, and merge images are shown on the right panel. Coated microorganisms with PPP12 gave similar images.
tect such a sensitive strain (*L. delbrueckii* subsp. *bulgaricus* CIDCA 333) both during freeze-drying and storage.

5. Conclusion

Uncoated *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 is very sensitive to gastro-intestinal conditions even when freeze-dried in the presence of sucrose. The use of LbL encapsulation technique using CHI and PPP protected *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 against gastro-intestinal conditions, allowing the release of high bacterial concentrations in the gut, the desired location of release.

Considering the Generally recognized as safe (GRAS) status of CHI, PLL and PAA and the non-toxicity of microorganisms coated with CHI and PPP, the protocol developed in this work appears as an interesting strategy to deliver sensitive lactic acid bacteria strains with CHI and PPP, the protocol developed in this work appears as an interesting strategy to deliver sensitive lactic acid bacteria strains in functional foods and drinks (*e.g.*, juices), avoiding bacterial release in the food product.

As the correct delivery of bacteria in gut involves not only technological but also physiological aspects, the results obtained in this work represent a solid support for the development of novel strategies for probiotic delivery and for the development of novel functional foods.

Competing interests

The authors declare that they have no competing interests.

Author’s contributions

G.Q., M.G.S. and A.H. did the experimental work. M.G.S., P.A. and P.F. analyzed results regarding the synthesis and physical-chemical characterization of polymers. G.Q., E.G. and A.G.-Z. analyzed microbiological results. A.G.-Z., P.A., E.G., A.H., M.G.S. and P.N.S. participated in the writing of the manuscript. A.G.-Z and P.N.S. coordinated the work. All authors have approved the final version of the manuscript.

Acknowledgments

This work was supported by the Argentinean Agency for the Scientific and Technological Promotion (ANPCyT) (Projects PICT/2014/0912), the Ministry for Science and Technology and the Portuguese Science Foundation (MCPyT-FCT, project PO-14-05). E.G., A.H. and A.G.-Z. are members of the research career CONICET. G.Q. is doctoral fellow from CONICET, P.A. is postdoctoral fellow from FCT (SFRH/BPD/69410/2010).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jff.2017.06.007.

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