CrossMark

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

G. Quintana [a,1], M.G. Simões [b,1], A. Hugo [a], P. Alves [b], P. Ferreira [b], E. Gerbino [a], P.N. Simões [b,*], A. Gómez-Zavaglia [a]

[a] Center for Research and Development in Food Cryotechnology (CCT-Conicet La Plata, UNLP), RA-1900, Argentina
[b] CIEPOPF, Department of Chemical Engineering, University of Coimbra, Polo II, Pinhal de Marrocos, P-3030-790 Coimbra, Portugal

ARTICLE INFO

ABSTRACT

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was encapsulated by layer-by-layer (LbL) technique. Copolymers of pluronic and poly(acrylic acid), were synthesized and characterized, and chitosan was selected as cationic polyelectrolyte. The efficiency of encapsulation was evaluated by plate counting the microorganisms before and after coating, after freeze-drying with sucrose, and after exposure to simulated gastrointestinal conditions. Coated microorganisms with 2/3 layers were also protected against simulated gastro-intestinal conditions, while cultivability of uncoated bacteria significantly dropped. Confocal microscopy allowed determining the integrity and permeability of the coated and uncoated microorganisms. Coated bacteria were non-toxic to eukaryotic cells, by the MTT assay, and showed no significant decrease of cultivability before and after freeze-drying and after 60 days of storage at 4 °C.

Considering the GRAS status of the used polymers, the results obtained revealed a potential delivery system of sensitive lactic acid bacteria strains in food products, ensuring their safe arrival to the gut.

© 2017 Elsevier Ltd. All rights reserved.

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 nanostructurated 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, Fakhrullin, & 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 carboxyl methylcellulose as polyelectrolytes (Cook, Tzortzis, Khutoryanskiy, & Charalampopoulos, 2013; Decher et al., 1992; Kahraman et al., 2009; Priya et al., 2011; Tang, Wang, Podsiadlo, & 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; Kahraman et al., 2009; 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 (Seki & 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, & Gómez-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), nonane (Sigma-Aldrich, St. Louis, MO, USA), deuterated tetrahydrofuran (THF; d_8-THF) (Euriso-top, Saint-Aubin, France), sodium chloride (Sigma-Aldrich, St. Louis, MO, USA), sodium hydroxide (Panreac, Madrid, Spain), hydrochloric acid (Fisher Scientific, USA), potassium chloride (Anedra, Buenos Aires, Argentina), sodium bicarbonate (Biopack, Buenos Aires, Argentina), sodium hydrogen phosphate (Anedra, 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), pancreatin (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-cyanovaleric 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). ^{1}H NMR spectra were obtained using a Bruker Advance III 400 MHz spectrometer 9.4 T (Karlsruhe, Germany). Samples were dissolved in d_8-THF, and analyzed in tubes of 5 mm diameter, using tetramethylsilane as internal standard.
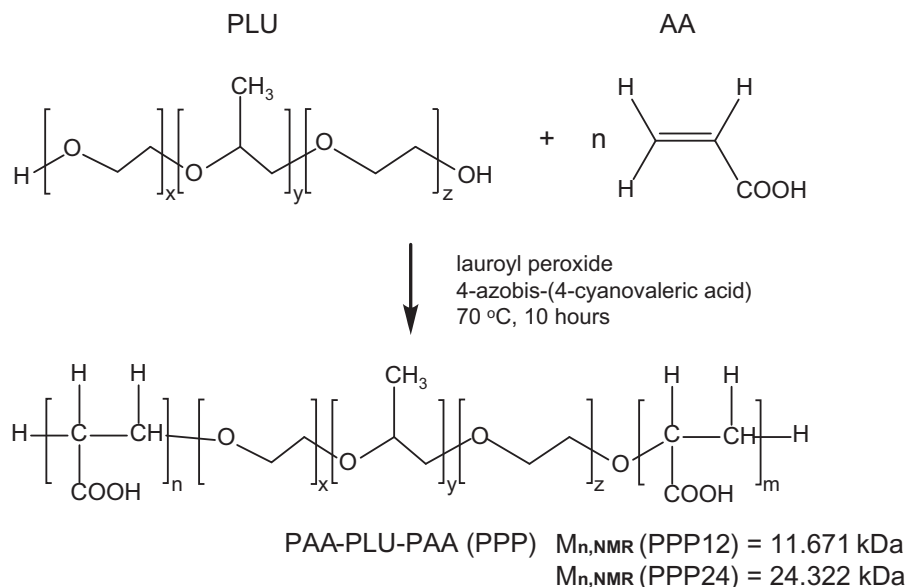
Fig. 1. Schematic representation of the synthesis of PAA-PLU-PAA (PPP). x = number of ethylene oxide (EO) units, y = number of O—CH₃—CH₂ units, z = number of propylene oxide (PO) units, m, n = number of acrylic acid units.

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 ($\sim 5 \times 10^8$ 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 10^7–10^8 CFU/mL. The deposition of the first layer was carried out at 37 °C under gentle stirring at 200 rpm for 1 h [(CHI/PPP)1/0]. The excess of CHI was removed by centrifugation (6000 rpm, 5 min, 4 °C) and microorganisms were washed twice with 0.15 M NaCl. The second layer was deposited by adding 20 mL PPP to the one-layered microorganisms, and then incubating at 37 °C under gentle stirring at 200 rpm (MaxQ 4000, Thermo Scientific, NJ, USA) for 1 h, [(CHI/PPP)1/1]. The procedure of alternating polymer adsorption was repeated to obtain microorganisms coated with two layers of chitosan and a layer of PPP, [(CHI/PPP)2/1], and with two layers of each polyelectrolyte, [(CHI/PPP)2/2].

To ensure the self-assembly deposition of the layers, the pH of all solutions was adjusted to 6. At this pH the amino groups of CHI were protonated (pKa = 6.5) and the carboxyl groups of PLU were deprotonated (pKa = 4.95), thus leading to positive and negative charges on the polyelectrolyte chains.

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 10^8 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 $CaCl_2$, 98 mM NaCl, 13.6 mM KCl, 0.82 mM KH_2PO_4, 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 K_2HPO_4; 98 mM NaCl; 5.5 mM Na_2HPO_4). 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 $NaHCO_3$, 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 1×10^5 cells per well and incubated for 5 days. Afterwards, coated and uncoated microorganisms were added at a concentration of 1×10^8 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 mg/mL MTT. After 3 h of incubation, 0.2 mL dimethyl sulphoxide was added to remove formazan from cells and absorbance was recorded at 560 nm (Synergy HT microplate reader, Bio-Tek Instruments, Vermont, USA). Cell viability was determined according to Eq. (1):

$$\text{Cell viability } (\%) = (OD_t)/(OD_c) \times 100\% \tag{1}$$

where OD_t is the optical density of the treated cells (with uncoated and coated microorganisms) and OD_c is the optical density of the control cells (non-treated cells).

2.2.9. Statistical analysis

All the assays were conducted in triplicate and in three independent assays. Average values were used for data analysis. Analysis of variance (ANOVA) was carried out for all the assays, using the statistical program InfoStat 2008 (Infostat Group/FCA. National University of Cordoba. Ed. Brujas, Cordoba, Argentina). Comparison of means was tested using Tukey methods, and if p < 0.05, the difference was considered statistically significant.

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 synthetized 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 ^{1}H NMR (Fig. 2). As both the ATR-FTIR and ^{1}H NMR spectra of PLU F68 and F127 were similar, only one of them is shown. Fig. 2A (1) shows the ^{1}H 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—C*H₃*), and at 1.6 ppm the terminal —O*H* 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 (—C*H*—CH₂) of the AA units at 2.2–2.8 ppm (peak *g* in the figure) and the methyl hydrogen of PLU segment (—CH—C*H₃*) 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 ascribed 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 carbonyls 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 νCOC 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):

$$Abs(t) = \frac{Abs_f - Abs_0}{1 + {}^t c^p} + Abs_0 \tag{2}$$

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
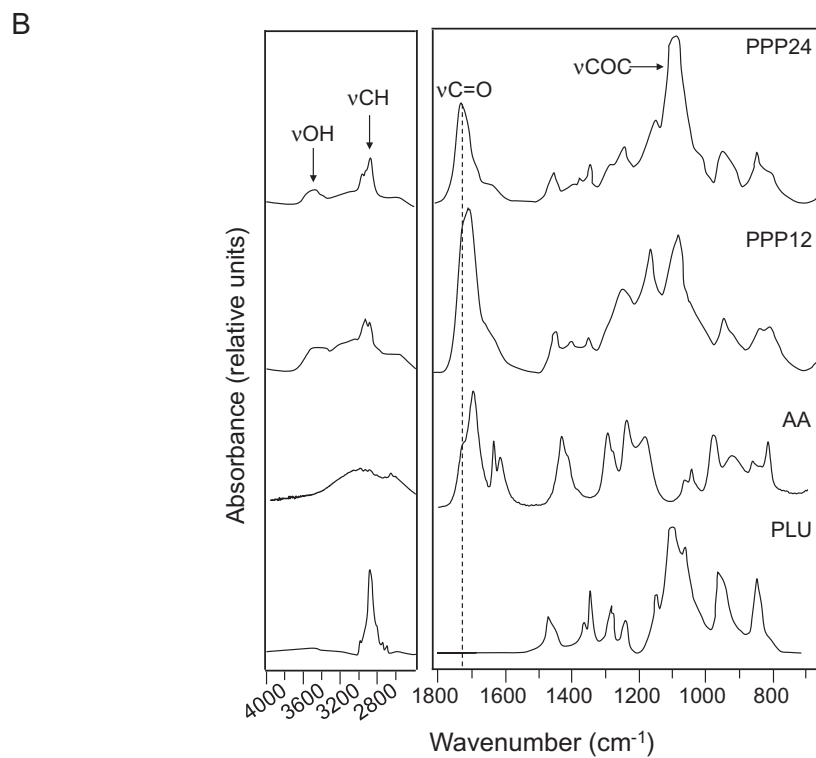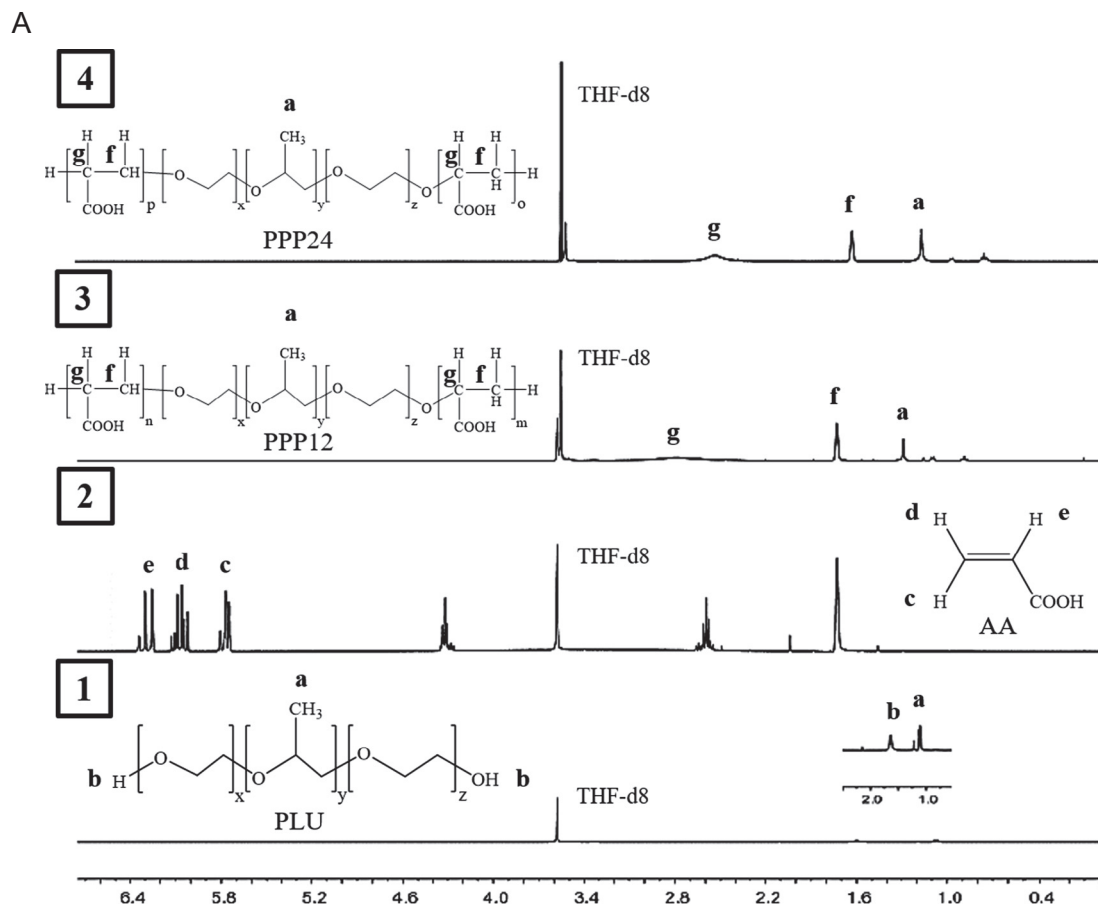
Fig. 2. (A) ^{1}H NMR spectra of PLU (1), AA (2), PPP12 (3) and PPP24 (4) in THF-d$_8$ at 400 MHz. (B) ATR-FTIR spectra of PLU, AA, PPP12 and PPP24.
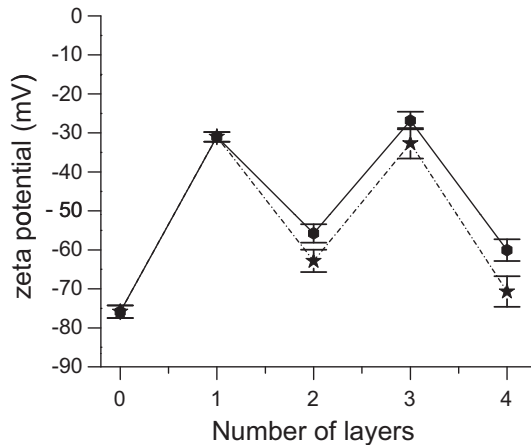
Fig. 3. Zeta potential of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 after the addition of different numbers of CHI and PPP layers.

Table 1
Kinetic parameters of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 grown in MRS broth after adding each layer of CHI and PPP.

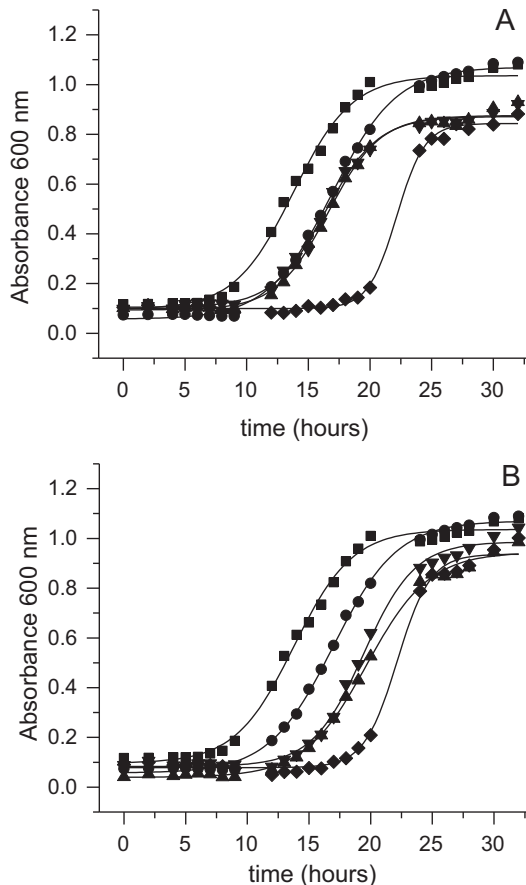| Coating	*lag* time (h)	Growth rate (absorbance units/h)	R^2
Non-coated	8.4	0.18	0.995
CHI/PPP12			
CHI/PPP 1/0	11.2	0.17	0.999
CHI/PPP1/1	12.0	0.21	0.996
CHI/PPP2/1	12.0	0.20	0.996
CHI/PPP2/2	19.5	0.38	0.997
CHI/PPP24			
CHI/PPP1/0	11.2	0.17	0.999
CHI/PPP1/1	14.3	0.17	0.997
CHI/PPP2/1	14.8	0.21	0.996
CHI/PPP2/2	19.5	0.32	0.995

Fig. 4. Growth kinetics of uncoated and coated *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 grown in MRS. Squares: uncoated bacteria, circles: bacteria coated with one layer of CHI [(CHI/PPP)1/0]; up triangles: bacteria coated with one layer of CHI and one of PPP [(CHI/PPP)1/1]; down triangles: bacteria coated with two layers of CHI and one of PPP [(CHI/PPP)2/1]; diamonds: bacteria coated with two layers of CHI and two of PPP [(CHI/PPP)2/2]. Full lines indicate the mathematical regressions according to Eq. (2) (see text). (A) Bacteria coated with CHI and PPP12 layers. (B) Bacteria coated with CHI and PPP24 layers.

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

PPP12 and 14.3 or 14.8 when using PPP24. The third profile was that of bacteria coated with [(CHI/PPP)2/2], 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/PPP)2/2] 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)1/0], [(CHI/PPP12)1/1] and [(CHI/PPP12)2/1] 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]1/1] showed a higher intensity of the red stain in regard with bacteria coated with [CHI/PPP12]2/1] (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.
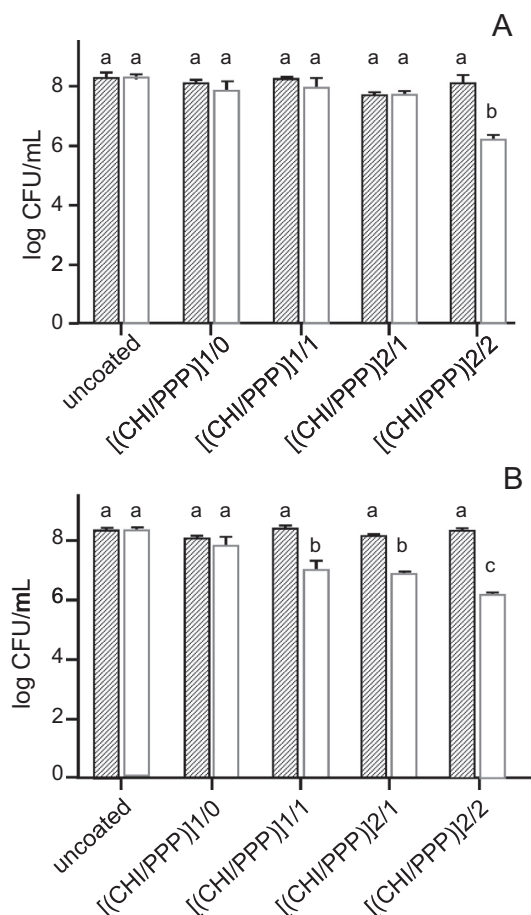
Fig. 5. Log CFU/mL of uncoated and coated *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 before and after freeze-drying in the presence of 20 % w/v sucrose. Slashed bars denote cultivability before the process and white bars, after the process. Statistical analysis was carried out by two-way ANOVA, and 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.

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
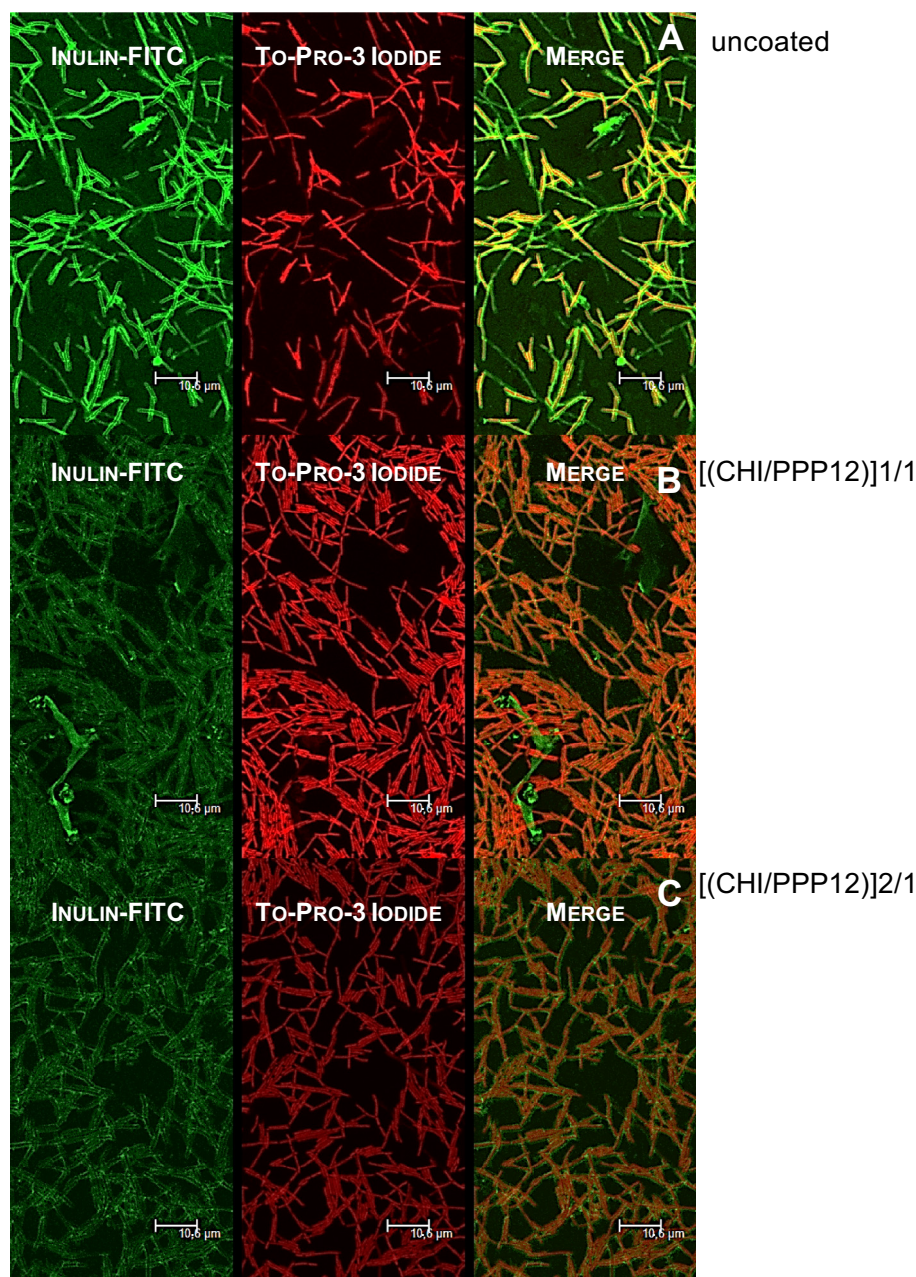
Fig. 6. Confocal laser scanning micrographs of uncoated (A) and coated (B, C) *L. delbrueckii* subsp. *bulgaricus* CIDCA 333. Coated cells correspond to [(CHI/PPP24)1/1] (B) and [(CHI/PPP24)2/1] (C) cells. Green fluorescence is shown on the left panel, red fluorescence is shown in the middle, and merge images are shown on the right panel. Coated microorganisms with PPP12 gave similar images.

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-
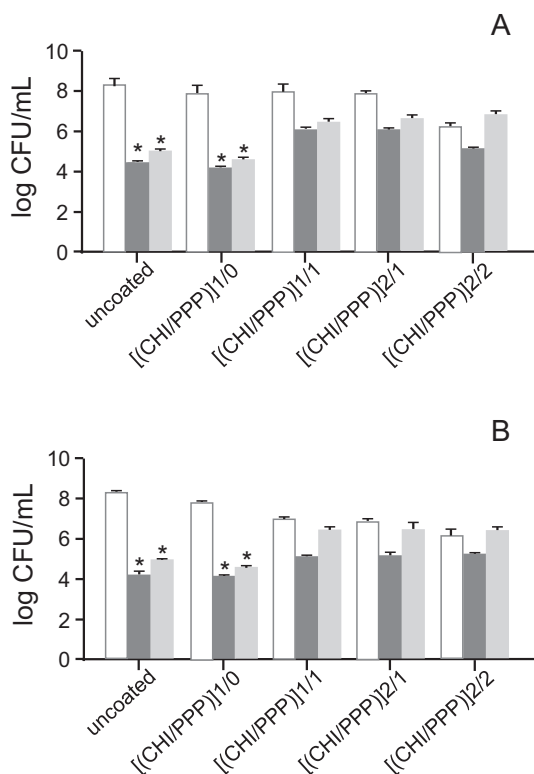
Fig. 7. Log CFU/mL of freeze-dried uncoated and coated *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 after exposure to gastric and intestinal conditions. White bars represent the CFU/mL of freeze-dried microorganisms, before exposure to gastric and intestinal conditions. Gray bars denote freeze-dried microorganisms after exposure to gastric conditions, and black bars, to freeze-dried bacteria after exposure to intestinal conditions. ANOVA was performed to compare cultivability before and after exposure to gastric and intestinal conditions. Asterisks denote significant differences within each group of values (p < 0.05). (A) Bacteria coated with CHI and PPP12 layers. (B) Bacteria coated with CHI and PPP24 layers.

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, PLU 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 in food products. Moreover, the resistance of coated bacteria to acidic pH becomes an important advantage for their use in acidic 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.

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.

References

Alakhov, V., Pietrzynski, G., Patel, K., Kabanov, A., Bromberg, L., & Hatton, T. A. (2004). Pluronic block copolymers and Pluronic poly(acrylic acid) microgels in oral delivery of megestrol acetate. *Journal of Pharmacy and Pharmacology, 56*, 1233–1241.

Alves, P., Hugo, A. A., Tymczyszyn, E. E., Ferreira, A. F., Fausto, R., Pérez, P. F., ... Gómez-Zavaglia, A. (2013). Effect of cholesterol-poly(N,N-dimethylaminoethyl methacrylate) on the properties of stimuli-responsive polymer liposome complexes. *Colloids and Surfaces B Biointerfaces, 104*, 254–261.

Barreiro-Iglesias, R., Bromberg, L., Temchenko, M., Hatton, T. A., Alvarez-Lorenzo, C., & Concheiro, A. (2005). Pluronic-g-poly(acrylic acid) copolymers as novel excipients for site specific, sustained release tablets. *European Journal of Pharmaceutical Sciences, 26*, 374–385.

Chandaroy, P., Sen, A., & Hui, S. W. (2001). Temperature-controlled content release from liposomes encapsulating Pluronic F127. *Journal of Control Release, 76*, 27–37.

Choo, E. S. G., Yu, B., & Xue, J. (2011). Synthesis of poly(acrylic acid) (PAA) modified Pluronic P123 copolymers for pH-stimulated release of doxorubicin. *Journal of Colloids and Interface Sciences, 358*, 462–470.

Coimbra, P., Alves, P., Valente, T. A. M., Santos, R., Correia, I. J., & Ferreira, P. (2011). Sodium hyaluronate/chitosan polyelectrolyte complex scaffolds for dental pulp regeneration: Synthesis and characterization. *International Journal of Biological Macromolecules, 49*, 573–579.

Cook, M. T., Tzortzis, G., Khutoryanskiy, V. V., & Charalampopoulos, D. (2013). Layer-by-layer coating of alginate matrices with chitosan–alginate for the improved survival and targeted delivery of probiotic bacteria after oral administration. *Journal of Materials Chemistry B, 1*, 52–60.

de Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*, 130–135.

Decher, G., Hong, J. D., & Schmitt, J. (1992). Buildup of ultrathin multilayer films by a self-assembly process: III. Consecutively alternating adsorption of anionic and cationic polyelectrolytes on charged surfaces. *Thin Solid Films, 210*, 831–835.

Gomez-Zavaglia, A., Abraham, A. G., Giorgieri, S., & De Antoni, G. L. (1999). Application of polyacrylamide gel electrophoresis and capillary gel electrophoresis to the analysis of *Lactobacillus delbrueckii* whole-cell proteins. *Journal of Dairy Science, 82*, 870–877.

Grimoud, J., Durand, H., Courtin, C., Monsan, P., Ouarné, F., Theodorou, V., & Roques, C. (2010). *In vitro* screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics. *Anaerobe, 16*, 493–500.

Guzmán, E., Cavallo, J. A., Chuliá-Jordán, R., Gomez, C., Strumia, M. C., Ortega, F., & Rubio, R. G. (2011). PH-induced changes in the fabrication of multilayers of poly (acrylic acid) and chitosan: Fabrication, properties, and tests as a drug storage and delivery system. *Langmuir, 27*, 6836–6845.

Kabanov, A. V., Batrakova, E. V., & Alakhov, V. Y. (2002). Pluronic (R) block copolymers as novel polymer therapeutics for drug and gene delivery. *Journal of Control Release, 82*, 189–212.

Kahraman, M., Zamaleeva, A. I., Fakhrullin, R. F., & Culha, M. (2009). Layer-by-layer coating of bacteria with noble metal nanoparticles for surface-enhanced Raman scattering. *Analytical and Bioanalytical Chemistry, 395*, 2559–2567.

Ma, W., Xu, H., Nie, S., & Pan, W. (2008). Temperature-responsive, Pluronic-g-poly (acrylic acid) copolymers in situ gels for ophthalmic drug delivery: Rheology,

in vitro drug release, and in vivo resident property. *Drug Development and Industrial Pharmacy, 34*, 258–266.

Priya, J., Vijayalakshmi, S. P., & Raichur, A. M. (2011). Enhanced survival of probiotic *Lactobacillus acidophilus* by encapsulation with nanostructured polyelectrolyte layers through layer-by-layer approach. *Journal of Agricultural and Food Chemistry, 59*, 11838–11845.

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science, 31*, 603–632.

Romano, N., Schebor, C., Mobili, P., & Gomez-Zavaglia, A. (2016). Role of mono- and oligosaccharides from FOS as stabilizing agents during freeze-drying and storage of *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Food Research International, 90*, 251–258.

Seki, K., & Tirrell, D. A. (1984). PH-dependent complexation of poly(acrylic acid) derivatives with phospholipid vesicle membranes. *Macromolecules, 17*, 1692–1698.

Tang, Z., Wang, Y., Podsiadlo, P., & Kotov, N. A. (2006). Biomedical applications of layer-by-layer assembly: From biomimetics to tissue engineering. *Advance Materials, 18*, 3203–3224.

Tymczyszyn, E. E., Díaz, M. R., Gómez-Zavaglia, A., & Disalvo, E. A. (2007). Volume recovery, surface properties and membrane integrity of *Lactobacillus delbrueckii*

subsp. *bulgaricus* dehydrated in the presence of trehalose or sucrose. *Journal of Applied Microbiology, 103*, 2410–2419.

Tymczyszyn, E. E., Díaz, M. R., Pataro, A., Sandonato, N., Gómez-Zavaglia, A., & Disalvo, E. A. (2008). Critical water activity for the preservation of Lactobacillus bulgaricus by vacuum drying. *International Journal of Food Microbiology, 128*, 342–347.

Tymczyszyn, E. E., Gerbino, E., Illanes, A., & Gómez-Zavaglia, A. (2011). Galacto-oligosaccharides as protective molecules in the preservation of *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Cryobiology, 62*, 123–129.

Tymczyszyn, E. E., Sosa, N., Gerbino, E., Hugo, A. A., Gómez-Zavaglia, A., & Schebor, C. (2012). Effect of physical properties on the stability of *Lactobacillus bulgaricus* in a freeze-dried galacto-oligosaccharides matrix. *International Journal of Food Microbiology, 155*, 217–221.

Yucel Falco, C., Sotres, J., Rascon, A., Risbo, J., & Cardenas, M. (2017). Design of a potentially prebiotic and responsive encapsulation material for probiotic bacteria based on chitosan and sulfated beta-glucan. *Journal of Colloids and Interface Surfaces, 487*, 97–106.

Zheng, H., Gao, M., Ren, Y., Lou, R., Xie, H., Yu, W., ... Ma, X. (2017). An improved pH-responsive carrier based on EDTA-Ca-alginate for oral delivery of *Lactobacillus rhamnosus* ATCC 53103. *Carbohydrate Polymers, 155*, 329–335.