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Microencapsulation of *Lactobacillus casei* and *Lactobacillus rhamnosus* in pectin and pectin-inulin microgel particles: Effect on bacterial survival under storage conditions

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ABSTRACT

The main objective of the research was to evaluate the performance of synbiotic delivery systems using pectin microgels on the protection of two probiotic strains (*Lactobacillus casei* ATCC 393 and *Lactobacillus rhamnosus* strain GG [ATCC 53103]) to simulated gastrointestinal digestion (GD) and storage conditions (4 ± 1 °C) in a 42 days trial. Microgel particles were prepared by ionotropic gelation method and three variables were evaluated: incubation time (24 and 48 h), free *vs* encapsulated cells, and presence or absence of prebiotic (commercial and Jerusalem artichoke inulin). Results demonstrated an encapsulation efficiency of 96 ± 4% into particles with a mean diameter between 56 and 118 μm. The viability of encapsulated cells after 42 days storage stayed above 7 log units, being encapsulated cells in pectin-inulin microgels more resistant to GD compared to non-encapsulated cells or without prebiotics. In all cases incubation time influenced the strains' survival.

1. Introduction

In the last 40 years people's awareness of the importance of an integral well-being has significantly increased, therefore, consumers are more often interested in food products having a positive impact on their health. For this reason, functional foods are among the most demanded ones by health-conscious consumers and their production is being recognized as the number one food biotechnology industry [1]. Nowadays, foods containing probiotic bacteria have attracted attention due to the wide range of health benefits associated to their consumption, compared to traditional food products [2]. However, the viability of probiotics cells might be negatively affected during food processing and storage. There are many extrinsic (temperature, relative humidity, and gaseous atmosphere) and intrinsic (nutrients, pH, acidity, and oxidation–reduction potential) factors which affect the viability and stability of probiotics in carrier foods [3]. Furthermore, to fulfill their crucial role, probiotics must survive in the acidic conditions of the stomach and be delivered to the intestines in high numbers [4].

Immobilization technologies, such as microencapsulation (ME), have been extensively used to solve these drawbacks by enhancing the survival of cells against harsh gastrointestinal (GI) conditions while increasing the stability and viability of probiotic cells by reducing their contact with the physicochemical stressors [5]. Alginate is still the most used wall material to entrap probiotics, however, a growing interest has been observed regarding the total or partial substitution of this anionic polysaccharide by other polysaccharides obtained from natural sources, such as plants. Pectin is an anionic heteropolysaccharide with a linear primary structural feature of α 1,4 linked D-galacturonic acid chain with varying degrees of methylation. A recent interest has arisen in the commercial use of pectin due to their long-standing reputation for being non-toxic or generally considered safe, with relatively low production costs and high availability. Citrus peels and apple pomace form the prime sources of pectin for commercial usage [6], waste streams from fruits and vegetables both present propitious sources of pectin for functional and nutritional use. Hence, sources other than conventional ones are being looked into to achieve waste valorization [7]. Moreover, because the gelling mechanisms are relatively simple, there is an interest in the preparation of hydrogels as carriers for probiotics

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encapsulation. Furthermore, as an added advantage, pectin shows lesser sensitivity to chemical agents and higher resistance to gastric environments than alginate, and secondly as an anionic polysaccharide, pectin has been reported as a good mucoadhesive material [8], which may prolong the residence and the exposure time. Pectin presents as aggregates of macromolecules in acid environments and is resistant to proteases and amylases, which are active in the upper GI tract, but digested by part of the colonic microbiota. Because of these unique characteristics, pectin has been successfully used for the construction of several oral delivery systems for controlled drug delivery to the colon [9,10].

The inclusion of compounds with prebiotic activity to the coating matrix, has been reported as an approach leading to higher encapsulation yield, increased resistance of co-encapsulated bacteria to GI stress [11], and to favor their competition with the resident intestinal microbiota [12]. Inulin has drawn much attention as an ingredient in the development of functional foods given its technological properties and its beneficial effects on health. It is a natural dietary soluble fiber which consists of a mixture of oligo- and/or polysaccharides of β (2 → 1) linked D-fructose units with a terminal glucose residue linked by α (1 → 2) bond [14]. At an industrial level, it is mainly obtained from chicory but Jerusalem artichoke (*Helianthus tuberosus* L.) has lately been used as a valuable alternative source [13].

On the other hand, the intrinsic physiological state of the probiotic cultures can also be a major factor affecting the overall culture viability [14] and its tolerance response to different stress situations, hence influencing the effect once in the host intestine. It has previously been suggested [15] that the growth phase during which probiotic bacteria are harvested can strongly influence their performance not only in the final product but also the physiological response in the host and also the success of the previously mentioned strategies. Hence, when aiming to optimize the health-promoting properties of probiotic bacteria, it is essential to determine the most favorable growth conditions and phase for cell harvesting.

The *Lactobacillus casei* group (LCG), comprised mainly of the closely related *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* species, are among some of the most studied and applied probiotic species of lactobacilli mainly due to their commercial, industrial and applied health promoting potential [16]. The group remains of interest as probiotics, and their use is widespread in industry. Commercially, they are used to ferment dairy products, often producing foods with improved flavor and texture. They have also been found to produce many bioactive metabolites which can confer benefits to the host when consumed [17]. As such, many LCG strains are considered to be probiotics. One member, *L. rhamnosus* strain GG (ATCC 53103), is perhaps one of the most studied bacterial strains in relation to health applications [18]. Likewise, several strains of *L. casei* are considered to be probiotics. Among them, *L. casei* ATCC 393 is a well-known probiotic strain with many applications in food production. It has been successfully used as starter or adjunct culture for the production of several dairy products, such as fermented milk, yogurt and cheese [19–21]. In addition, it has been associated with several health promoting activities like regulation of intestinal microbiota [22], tumor-inhibition, pro-apoptotic and anti-proliferative effects [23] and recently with the production of bioactive peptides in fermented milk [19].

In view of the above, the main objective of this work was to perform a synbiotic delivery system model that evaluated and compared the survival of two probiotic strains within the three variables previously described: encapsulation in pectin hydrogels, presence of inulin as prebiotic and different harvesting times. To the best of our knowledge, the binary interaction between pectin synbiotic matrix with inulin of different origins and the impact of microbial harvesting time on the survival of *Lactobacillus casei* and *Lactobacillus rhamnosus* to GI challenge and storage conditions has not been reported yet for probiotic enhanced delivery.

2. Material and methods

2.1. Microorganisms and culture conditions

The strains used in the study were *Lactobacillus casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 53103 (strain GG onwards *L. rhamnosus* 53103) both purchased from the American Type Culture Collection (ATCC, Rockville, MD). Stock cultures of the strains were stored at −70 °C in 20% (v v^{-1}) glycerol until use. Prior to its use, each strain was suspended in De Man, Rogosa and Sharpe broth (MRS, Biokar Diagnostics, Beauvais, France), incubated at 37 ± 1 °C, harvested by centrifugation at 5000 ×*g* for 10 min (Labofuge 200, Kendro, Germany) and cell pellets were subsequently washed twice with sterile phosphate-buffered saline (PBS, pH 7.2).

Three variables were evaluated: incubation time, presence or absence of prebiotic (inulin) and free *vs* encapsulated cells. In order to evaluate the effect of incubation time on the strains survival capability, both strains were harvested after 24 and 48 h incubation. For the assessment of the effect of presence *vs* absence of prebiotic, washed cell pellets were re-suspended in PBS and PBS + prebiotic, whereas for the evaluation of free *vs* encapsulated cells, these bacterial suspensions were divided into two parts: one used for encapsulation and the other as free cells for control purposes.

2.2. Materials

Inulin was the selected carbohydrate to study its prebiotic activity in the configuration of the pectin synbiotic matrix. For this purpose, food grade commercial inulin (CI) with a carbohydrate content ≥ 90 g/100 g (kindly donated by Granotec S.A., Santiago, Chile) and Jerusalem artichoke inulin (JAI, kindly donated by Dr. Irene Rubel from the Universidad Nacional del Centro de la Provincia de Buenos Aires, Argentina) obtained from *H. tuberosus* L. tubers, were used. For the microencapsulation technique, low methoxyl pectin Genu Pectin LM104 AS was used (CPKelco, Limeira, Brazil). According to the manufacturer, the pectin used had an esterification degree of 27%, and 20% of the original carboxyl groups were replaced by amide groups. Sunflower oil (Molinos Rio de la Plata, SA, Argentina) was used as dispersing phase, containing 2% wt Tween 80 (Merck KGaA, Darmstadt, Germany), and calcium chloride (Anerda S.A., San Fernando, Buenos Aires, Argentina) as cross-linking agent.

2.3. Preparation of synbiotic pectin hydrogels

For the preparation of the synbiotic hydrogels, first suspensions of *L. casei* 393 and *L. rhamnosus* 53103 were prepared in PBS (in order to obtain pectin beads + probiotic) and PBS with 1% (w v^{-1}) CI and JAI (in order to obtain pectin beads + probiotic + prebiotic) as it is described in Section 2.1. Inulin solutions were sterilized through 0.2 μm filters (Gamafil S.A., Argentina) before use and the final bacterial concentration was determined by plate count on MRS agar (Biokar Diagnostics, Beauvais, France).

Pectin microbeads were loaded with each *Lactobacillus* strain (with or without inulin) by ionotropic gelation method using $CaCl_2$ solution (crosslinking agent) assisted with w/o emulsion to decrease the particle sizes below 100 μm. Homogeneous particles with the proper mechanical strength and permeability barrier of hydrogels were reached optimizing the concentration of Ca^{+2} solution up to 0.5 wt% and improving the contacting method between the biopolymer and the crosslinker. As shown in Fig. 1, two w/o emulsions (30 mL) were prepared with the same water to oil ratio (w/o = 1) and 2 wt% of Tween 80 concentration for stabilization, the first one containing a pectin solution and the other one containing the $CaCl_2$ solution. In order to obtain the first emulsion, a pectin solution (2% wt) was prepared in distilled water at 50 °C under magnetic stirring at 300 rpm for 2 h. The solution was left to cold down until 30 °C measuring the pH at this point (~3.4), and 5 g of the probiotic solution (~10^{10} CFU mL^{-1}) were added. The mixture was stirred for

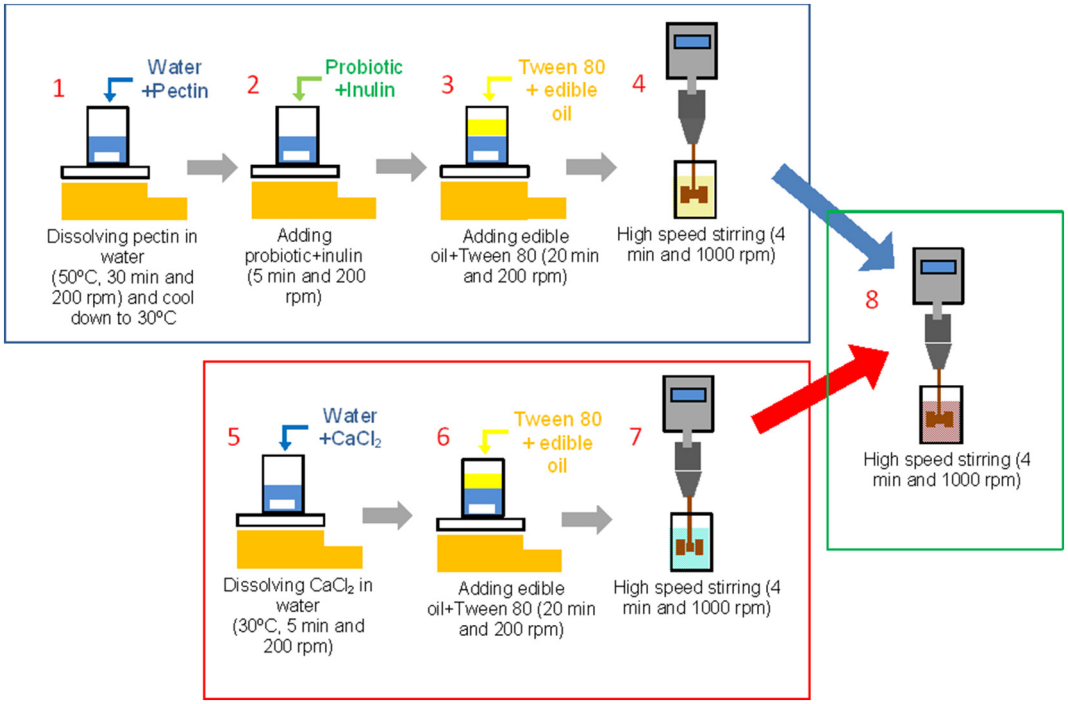

Fig. 1. Schematic representation of the inotropic gelation method used to encapsulate *Lactobacillus casei* 393 and *Lactobacillus rhamnosus* 53103 into pectin microbeads.

another 30 min before 15 mL oil phase with surfactant was incorporated reaching a final concentration of 2% wt Tween 80. The w/o emulsion was generated using a mixer (Ultra Turrax IKA I25) provided with a four-pitched blade impeller with a rod diameter of 4 mm and blades of 10 × 20 mm pitched at 45° to rotate at 1000 rpm during 4 min. The second emulsion, containing $CaCl_2$ solution at 0.5% (w w^{-1}) was prepared in the same way as the one containing the synbiotic microgels (pectin and probiotics, with or without prebiotic). Microparticles were generated by adding 30 g of $CaCl_2$ emulsion dropwise for 4 min to 30 g of the pectin-probiotic(-prebiotic) emulsion under stirring at 1000 rpm. After this, the resulting emulsion containing the microencapsulated probiotic cells was left stirring for another 30 min at 100 rpm.

The resulting microcapsules were recovered by a double washing-filtering sequence (Whatman® Grade 3 filter paper, provided by Sigma-Aldrich) by diluting 60 g of the final emulsion with 250 mL of distilled water containing 0.1% (v v^{-1}) Tween 80.

2.3.1. Microparticle's characterization

Particle size distribution of the pectin microgels was determined by analyzing digital micrographs of the emulsions with the software Image J 1.52a (National Institute of Health [NIH], Bethesda, MD, USA). Digital micrographs were taken by a Primo Star Zeiss microscope with an Axiocam ERc 5s camera. The images were converted to grayscale (8-bit) and binarized (converted from grey level to black and white) using a thresholding method to differentiate the pectin microspheres. The software determines the projected area of each particle and its diameter (d_i) was calculated considering the particles to be spherical (as evidenced by the micrographs). Hundreds of particles were detected in each micrograph and at least 3 micrographs were analyzed per sample so that more than 1000 particles were used to determine the particle size distribution (PSD) of each sample.

Since the volume occupied by the particles is relevant for probiotics ME, then the volume or mass moment (or De Brouckere) mean diameter (D_{43}) of each PSD was calculated:

$$D_{43} = \frac{\sum d_i^4}{\sum d_i^3}$$

As a measure of the width of the PSD, the standard deviation relative to D_{43} was calculated as:

$$Sd_{43} = \sqrt{\frac{\sum (d_i - D_{43})^2}{N}}$$

where N is the total number of particles.

2.3.2. Encapsulation efficiency

Bacterial encapsulation efficiency was determined through the Entrapment Yield (EY), which is a combined measurement of the efficacy of entrapment and the survival of viable cells, as reported previously by Olivares, Soto [24]:

$$EY = \left(\frac{Log\ N}{Log\ N_0}\right) \times 100$$

where N is the number of viable entrapped cells released from the microparticles and N_0 the number of free cells added to the polymer mixture during their production. The enumeration of viable cells was carried out as stated in Section 2.7.

Fluorescent staining Live/Dead BacLight (Molecular Probes, Eugene, OR) kit was used to detect the location and viability of probiotics in the hydrogels. Briefly, the kit consists of a mixture of two nucleic acid-binding stains: SYTO 9 and propidium iodide (PI), both differing in their spectral characteristics and in their ability to penetrate viable bacterial cells. A stock solution of SYTO 9 and PI stains (0.75 μL) were prepared in a 1:1 ratio and mixed by pipetting with 250 μL of PBS, stained samples were left 30 min in dark before observation using an epifluorescence microscope equipped with filters to detect both stains.

2.4. Evaluation of the viability during storage

Synbiotic microbeads were stored under refrigeration temperature at 4 ± 1 °C and protected from light. The viability shelf life of probiotic cells was evaluated by the enumeration of viable cells (Section 2.7) every 7 days for a period of 42 days.

2.5. Survival to simulated GI digestion

The *in vitro* evaluation of the resistance of the pectin and pectin-inulin hydrogel beads to simulated gastric fluid (SGF) and simulated intestinal fluids (SIF) was performed after 42 days of storage under refrigeration conditions. A two-step model was used as suggested by Banerjee, Chowdhury [25]; first, bacteria cells (1 g) were inoculated in SGF by using a sterile solution composed of 9 g L^{-1} NaCl and 0.3% (w/v) pepsin (Saporiti S.A., Argentina), pH was adjusted to 2.0 with HCl (0.1 M). Samples were incubated under low agitation at 37 °C and aliquots for cell counts were taken at t 0, 60 and 120 min. Secondly, samples were mixed with SIF composed of 9 g L^{-1} NaCl, 1% (w v^{-1}) pancreatin (Saporiti S.A., Argentina) and 0.3% (w v^{-1}) bile salt at a pH adjusted to 8.0 using NaOH (0.1 M). Enumeration was done immediately after contact and after 60 and 180 min. For control, free cells were used, in which case 1 g of an adjusted suspension was employed following the protocol previously described.

At the end of each step (SGF and SIF), the percentage reductions were calculated as follows:

$$\% = \left(\frac{Log\ N_o - Log\ N_f}{Log\ N_o}\right) \times 100$$

where N_0 is the initial cell counts and N_f the final cell counts at each step.

2.6. Prebiotic activity score

The prebiotic activity score (Apreb) was determined using the equation reported by Huebner, Wehling [26] with modifications:

$$A_{preb} = \frac{(LogP_{24} - LogP_0)_{prebiotic}}{(LogP_{24} - LogP_0)_{glucose}} - \frac{(LogE_{24} - LogE_0)_{prebiotic}}{(LogE_{24} - LogE_0)_{glucose}}$$

where P is the growth (CFU mL^{-1}) of the probiotic bacteria at t 24 h and t 0 h, E is the growth (CFU mL^{-1}) of *E. coli* ATCC 25922 at t 24 h and t 0 h and the indicated subscript corresponds to the prebiotic under study (CI, JAI and pectin) or glucose as a positive control. Briefly, the assays were carried by adding 1% (p v^{-1}) of CI, JAI, pectin or glucose to tubes containing MRS carbohydrate-free basal medium (MRS-B) for *L. casei* and *L. rhamnosus* and M9 medium for *E. coli* ATCC 25922. After 24 h of incubation at 37 °C, samples were enumerated on MRS agar and TSA (Tryptic Soy Agar, Difco, Detroit, USA) for probiotic strains and *E. coli* respectively. The initial suspensions (~10^8 cells mL^{-1}) were prepared from an overnight culture of *L. casei* 393, *L. rhamnosus* 53103 grown in MRS broth and TSB (Tryptic Soy Broth, Difco, Detroit, USA) for *E. coli*, centrifuged at 5000 ×*g* for 10 min and the cell pellets properly washed twice with PBS.

2.7. Enumeration of free and encapsulated Lactobacillus cells

For enumeration of free *L. casei* 393 and *L. rhamnosus* 53103 cells samples were serially diluted with PBS solution, plated on MRS agar and incubated at 37 °C for 48 h. To determine the entrapment and viability of encapsulated probiotic cells, 0.1 g of pectin microbeads were suspended in 0.1 M sodium citrate buffer pH 6.2 for 20 min followed by gentle shaking (50 rpm) until complete dissolution at room temperature. Serial dilutions were performed and the bacterial content of the microbeads were determined by plating on MRS agar and incubated for 48 h. In the case of stressed cells, plates were kept up to 72 h. Results were expressed as either log10 CFU g^{-1} or mL^{-1}. The detection limit of quantification assays was 1 CFU g^{-1} or mL^{-1}.

2.8. Statistical analysis

All analyses were performed in triplicate under identical conditions in two independent trials and the results expressed as mean and standard deviation (mean ± SD). Data were analyzed using Analysis of Variance (ANOVA) followed by Tukey's multiple comparison tests when necessary. Statistical significance was established at $p < 0.05$ for all the comparisons.

3. Results and discussion

3.1. Preparation and characterization of encapsulated Lactobacillus in synbiotic pectin hydrogels

The ionotropic gelation methodology employed to encapsulate *L. casei* 393 and *L. rhamnosus* 53103 cells into pectin hydrogels presented a remarkable EE of 96 ± 4% with no significant cell reductions. Between 1324 and 3690 particles per sample were analyzed. Results of the PSD analysis are shown in Table 1. The particle size of all samples ranged between 0.25 μm (the minimum detected by the software) and 161 μm. Mean diameter (D_{43}) ranged between 56 and 118 μm and standard deviation (Sd_{43}) ranged between 50 and 113 μm, meaning a relatively broad PSD, in accordance with the selected technique [27]. There was no significant effect of strain, incubation time or inulin-type on D_{43}, meaning that the encapsulation method was not affected by these treatments. As outlined by Dimitrovski, Velickova [28] a uniform capsule diameter should be achieved so as not to alter the sensory qualities of the food product, while simultaneously delivering the proper doses of probiotic; however, the diameter must not adversely affect the viability of the microorganisms. In line with the results reported by Hansen, Allan-Wojtas [29], who stated that the diameter should be of at least 100 μm to prevent a considerable reduction of microbial viability when exposed to simulated gastric juices, the capsules obtained in the present study were of suitable dimensions and agree with the aforementioned requirements to maintain both viability and sensory properties. As shown in Fig. 2A and B optical micrographs show the smooth surface morphology of the beads and the successfully encapsulated *L. casei* 393 and *L. rhamnosus* 53103 cells inside the pectin microcapsules in accordance with the excellent EE reached.

One major challenge is to ensure that coating materials preserve the viability of probiotics under the harsh conditions of the upper GI tract, but on arrival to the colon, release the immobilized cells. Pectin presents good attributes as a coating material, it remains intact in the stomach and the small intestine while can be easily digested by pectinases generated by the colonic microbiota [30]. Sandoval-Castilla, Lobato-Calleros [31] analyzing the entrapment of *L. casei* LBC81 LYO in beads made with sodium alginate, amidated low-methoxyl pectin, and blends of both concluded that the addition of pectin was responsible for a further beneficial effect, by reducing the decaying rate of cells.

Table 1
Particle size distribution parameters of pectin gel microencapsulation particles: volume or mass moment mean diameter (D_{43}), standard deviation relative to D_{43} (Sd_{43}), maximum diameter (D_{Max}), and number of particles analyzed (N). JAI: Jerusalem artichoke inulin, CI: commercial inulin.

Strain	Inulin	Incubation time (h)	D_{43} (μm)	Sd_{43} (μm)	D_{Max} (μm)	N (−)
L. casei 393	0%	48	85.5	80.9	125.9	3458
		24	81.8	75.0	136.9	1956
	1% CI	48	56.2	51.0	90.7	3278
		24	80.2	75.4	118.5	2806
	1% JAI	48	87.2	80.8	122.2	2184
		24	90.8	86.7	126.9	2490
L. rhamnosus 53103	0%	48	118.1	113.4	160.8	3297
		24	89.0	79.9	141.7	1324
	1% CI	48	77.6	73.1	110.3	2899
		24	57.6	51.5	95.5	2677
	1% JAI	48	57.1	50.3	118.0	3690
		24	90.3	87.5	115.1	1771
Average			**80.9**	**75.4**	**121.9**	**31830***

* Total number of particles analyzed.

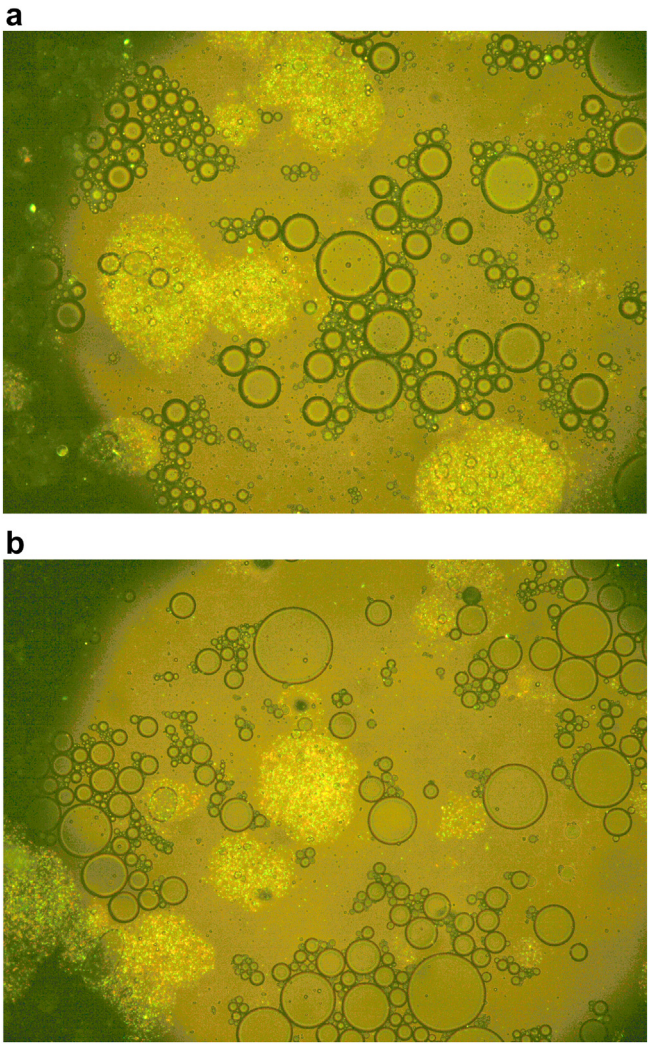

Fig. 2. Optical micrograph (with a 40× magnification objective) of (A) *Lactobacillus rhamnosus* 53103 and (B) *Lactobacillus casei* 393 cells entrapped in pectin microbeads stained with Live/Dead BacLight viability kit.

Moreover, low concentrations of Ca^{2+} ions were employed for the preparation of hydrogels to discourage the full gelation and formation of bulk gels [5]. It has been previously reported that amounts of Ca^{2+} ions higher than 0.5 M can lead to a competition for interaction with anionic sites COO^{-} which may result in weaknesses of gels [32], given that the intermolecular cross-links between the calcium ions (Ca^{2+}) and the negatively charged carboxyl groups (COO^{-}) promotes the gel network structure.

3.2. Assessment of the prebiotic activity

The viable counts of *L. casei* 393 and *L. rhamnosus* 53103 after incubation for 24 h with 1% of CI, JAI, pectin and glucose (included as positive control) are presented in Table 2. As evidenced by the results, all prebiotic tested could act as good growth substrates with no significant differences between CI and glucose.

When analyzing each substrate separately, the prebiotic activity scores obtained point towards pectin with better outcomes. As previously stated by Figueroa-Gonzalez, Rodríguez-Serrano [33], substrates with a high prebiotic activity score support good growth of probiotic bacteria comparable to glucose or better. However, the development of enteric bacteria, represented here by *E. coli* ATCC 25922, grown on the same prebiotic should be low compared to that on glucose. Overall, pectin was the supplement that most significantly enhanced the growth

Table 2
Viable cell count (Log CFU mL^{-1}) of probiotic strains and *E. coli* ATCC 25922 after 24 h incubation with Commercial inulin (CI), Jerusalem artichoke inulin (JAI), pectin and glucose. Results are given as media ± standard deviation.

Strain	Supplement			
	CI	JAI	Pectin	Glucose
L. casei 393	9.56 ± 0.10	9.18 ± 0.03	9.07 ± 0.10	9.53 ± 0.08
L. rhamnosus 53103	10.04 ± 0.18	9.30 ± 0.13	11.36 ± 0.15	10.15 ± 0.10
E. coli	8.85 ± 0.10	8.20 ± 0.15	8.51 ± 0.08	9.08 ± 0.20

of the probiotics compared to controls, with scores of 0.79 and 1.08 for *L. casei* 393 and *L. rhamnosus* 53103 respectively, followed by CI (0.17 and 0.12) and JAI (0.03 and 0.10). No significant differences ($p < 0.05$) were observed between CI and JAI compared to controls regarding the prebiotic activity, under the conditions tested. Similar results were obtained by Nazzaro, Fratianni [34] and Thomassen, Larsen [35] which recorded the prebiotic score of pectin for *L. plantarum* to be between 0.95 and 1.33, and of ~1.00 for *Bacillus coagulans*. For *B. coagulans* this behavior was related to the ability of the strain to produce polygalacturonase and pectin lyase for consumption of pectin while for *L. plantarum* it was stated that the presence of pectin had a considerably beneficial influence on the preservation of protein expression patterns if compared to the growth in presence of glucose used as control.

Given the known metabolic diversity of the lactobacilli, it might be expected a variation in the prebiotic activity scores for the different prebiotics used by a single probiotic strain and among strains within one prebiotic.

3.3. Survival of encapsulated Lactobacillus to storage

Storage viability of encapsulated *L. casei* 393 and *L. rhamnosus* 53103 cells at 4 °C was weekly monitored along 42 days trial. As stated by Raddatz, Poletto [36] during storage high probiotic viability is desirable since the number of viable probiotic bacteria in food products depends on the number of probiotics lost along processing and post-processing stages. In accordance with the excellent EE obtained, the viability of both *Lactobacillus* strains in presence of inulin (CI or JAI) after 42 days storage stayed above 7 log units, with a significant ($p > 0.05$) higher survival for older cultures (harvested at 48 h), compared to younger ones (24 h). Fig. 3A to D illustrates the viability of cell encapsulated in pectin based microparticles and after co-encapsulation with CI and JAI. In all cases, encapsulated cells retained the viability at levels considered appropriate by legislation with counts between 6.41 ± 1.26 and 10.58 ± 1.53 Log CFU g^{-1}.

As seen in Fig. 3A, for younger cultures of *L. casei* 393 (24 h) there were no significant differences ($p > 0.05$) between cells encapsulated in synbiotic hydrogels with CI and JAI after 42 days storage. The reductions registered were of 1.35 and 2.39 Log units respectively, while for cells encapsulated in pectin hydrogels (E1) reductions were of 3.43 Log units, significantly lower than cells co-encapsulated with the prebiotics ($p < 0.05$). On the other hand, as seen in Fig. 3B when analyzing 48 h cultures of *L. casei* 393 the presence of prebiotics (E2 and E3) did not result in an enhanced survival compared to E1 (final survival of 10.58 ± 1.53 Log CFU g^{-1}). No significant differences ($p > 0.05$) were evidenced among the presence of CI or JAI, reductions at the end of the 42 days were of 1.12 and 1.35 Log units respectively concerning to t_0.

When seen the behavior of *L. rhamnosus* 53103 in Fig. 3C and D at the end of the trial, 48 h cultures were significantly ($p < 0.05$) more resistant than 24 h ones in presence of CI and JAI than without any prebiotic, reductions were of 0.67 Log units for CI and 0.65 Log units for JAI *vs* 2.27 Log units reductions in absence of any prebiotic (Fig. 3D). The profile seen for 24 h cultures was slightly different, no statistical differences ($p > 0.05$) were seen among treatments (E1, E2, E3) even though CI (E2) lead to an increase in viability of 0.60 Log units in contrast with

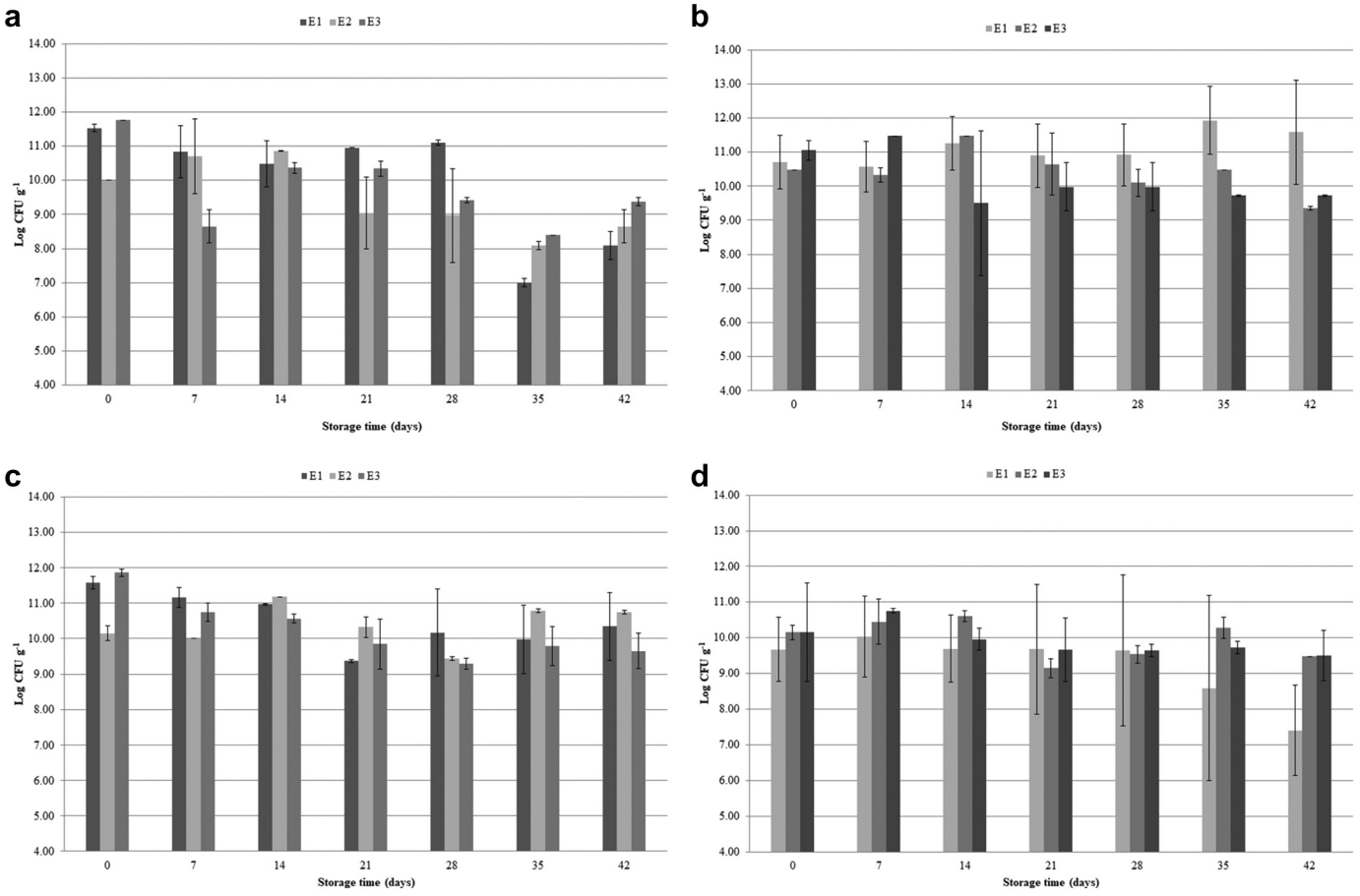

Fig. 3. Survival of encapsulated *Lactobacillus casei* (A: 24 h culture and B: 48 h culture) and *Lactobacillus rhamnosus* (C: 24 h culture and D: 48 h culture) cells in pectin beads without prebiotic (E1), pectin beads+CI (E2) and pectin beads+JAI (E3). Storage conditions: 4 °C for 42 days. Values are expressed as Log CFU g^{-1} media ± SD.

results seen for JAI (E3) or no prebiotic (E1), with drops of 2.20 and 2.15 Log units respectively (Fig. 3C).

3.4. Survival of free and encapsulated Lactobacillus to GI conditions

The ability to withstand the adverse conditions of the GI tract is an indispensable requirement for a microorganism to be classified as a probiotic of intestinal action. Fig. 4A–D shows the viability of free and microencapsulated *Lactobacillus* cells under sequential digestion. It was observed that the viability of encapsulated (E1, E2, E3) and non-encapsulated cells decreased throughout the GI simulation. When free *Lactobacillus* cells were sequentially incubated in SGF (M1) and SIF (M2) the survivability was reduced drastically ($p < 0.05$) in 91 and 98% for *L. casei* 393 and *L. rhamnosus* 53103 respectively, evidencing the sharper decline after the first contact hour with SFG.

In contrast, encapsulated cells had higher survival rates after the complete GI challenge and reductions along the gastric and intestinal phase were more progressive, indicating that synbiotic pectin beads improved considerably the survivability of both strains. As shown in Fig. 4A to D, cells were very sensitive to the first step (M1) under low pH conditions, compared to the behavior seen throughout the course of the second step (M2). Krasaekoopt, Bhandari [37] establish that when the synbiotic matrix absorbed bile salt in the intestine, an insoluble complex between calcium ion (positive) and cholate (negative) is formed electrostatically. This limits the diffusion of bile salt into the matrix and inhibits the interaction between the bile salt and entrapped cells. Meanwhile, Li, Zhang [38] postulates for *L. rhamnosus* GG that pectin in cross-linking with Ca^{2+} protects probiotic cells from acidic deactivation, in contrast to its free form making the encapsulated probiotic cells more "acid-resistant" than non-encapsulated.

The viability of free and encapsulated cells in absence of inulin decreased considerably, between 60 and 90%, after 120 min exposure (end of M1). Instead, the co-encapsulation in synbiotic pectin hydrogels with inulin significantly and efficiently ($p < 0.001$) improved its survival dimming the reduction percentages to 12–40% (M1) as evidenced in E2 and E3 of Fig. 4A to D. No significant differences ($p > 0.05$) were seen among CI and JAI concerning the protective effect exerted to both strains, except for *L. rhamnosus* 24 h cultures (Fig. 4C) in which counts in presence of JAI were significantly ($p < 0.01$) higher than with CI, 2.09 ± 0.12 and 5.66 ± 0.26 Log CFU g^{-1}, respectively. All in all, *L. rhamnosus* 53103 presented a better resistance profile compared to the ones seen for *L. casei* 393 except for 24 h cultures with CI.

When analyzing the behavior of the strains at the end of the sequential GI digestion (SGF + SIF), the survival of the strains varied according to the conditions tested. Among the three different capsules produced, E1 (beads without prebiotic) was the least effective in improving the survival of *Lactobacillus*, with counts below detectable level after exposure to sequential GI digestion for 24 h cell cultures; older cultures (48 h) were more resistant under the same conditions with counts of 2.52 ± 0.28 Log CFU g^{-1} for *L. casei* 393 and 0.50 ± 0.28 Log CFU g^{-1} for *L. rhamnosus* 53103, representing a 27 and 8% survival, respectively. Taking into consideration what was previously stated by Van Baarlen, Troost [39], who reported that consumption of probiotic bacteria harvested at different phases of growth has been shown to cause profoundly different mucosal responses in human and thus in its activity in the GIT in the present study was one of the variables under study.

As seen in Fig. 4B and D the results demonstrated that 48 h cultures of *L. casei* 393 and *L. rhamnosus* 53103 coated with pectin and co-encapsulated with inulin (CI and JAI) could further improve the viability over free cells and E1. At the end of the sequence, the viability of *L. casei*

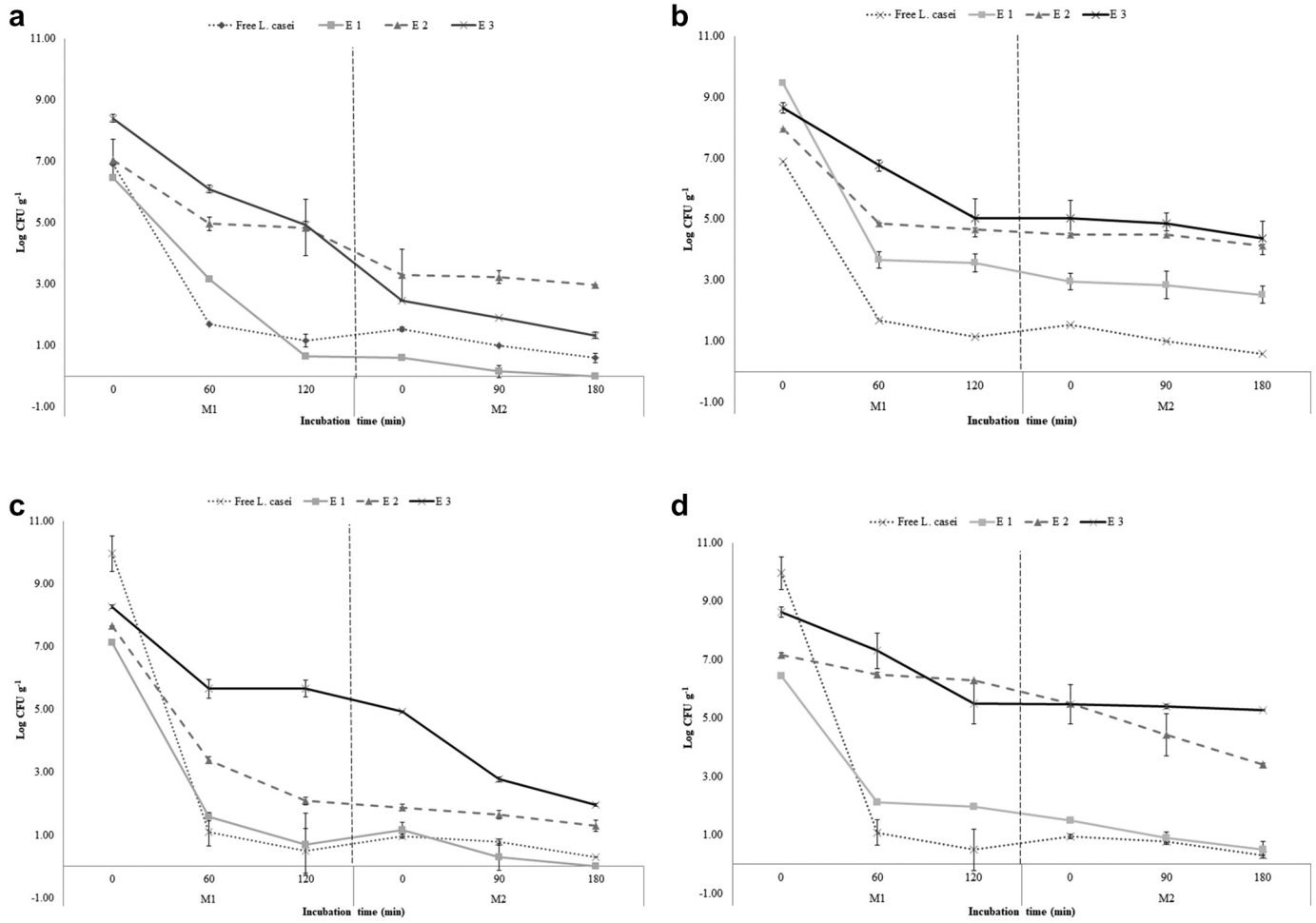

Fig. 4. Survival of free and encapsulated cells to simulated gastric fluids (M1) and simulated intestinal fluids (M2). (A) *Lactobacillus casei* - 24 h culture, (B) *Lactobacillus casei* - 48 h culture, (C) *Lactobacillus rhamnosus* - 24 h culture, (D) *Lactobacillus rhamnosus* - 48 h culture. E1: pectin beads without prebiotic, E2: pectin beads+CI, and E3: pectin beads+JAI. Results are expressed as Log CFU g^{-1} media ± SD.

393 + CI (E2) was of 4.13 ± 0.06 Log CFU g^{-1} and *L. casei* 393 + JAI (E3) 4.38 ± 0.55 Log CFU g^{-1} which represents a survival of 52 and 51%, respectively ($p > 0.05$). In the case of *L. rhamnosus* 53103 + CI (E2) survivability was of 3.42 ± 0.03 Log CFU g^{-1} and *L. rhamnosus* 53103 + JAI (E3) 5.28 ± 0.03 Log CFU g^{-1}, a 48 and 61% survival.

On the other hand the profiles of co-encapsulated 24 h cultures of both strains with inulin (Fig. 4A and C) evidence a lower survivability rate with counts of 2.97 ± 0.00 Log CFU g^{-1} for *L. casei* 393 + CI (E2) and 1.33 ± 0.10 Log CFU g^{-1} for *L. casei* 393 + JAI (E3), representing a 42 and 16% survival. For *L. rhamnosus* 53103 the results followed the same trend, 1.30 ± 0.17 Log CFU g^{-1} for CI (E2) and 1.96 ± 0.04 Log CFU g^{-1} for JAI (E3), a 17 and 24% survival.

The stability of probiotics during digestion is essential as the number of viable cells reaching the colon determines whether these probiotics can exert a healthful effect. As evidenced in the present study the behavior of 48 h cultures of both strains in presence of inulin (E2 and E3) towards M2 was stable along with the 180 min exposure compared to 24 h cultures. Similarly, Bepeyeva, de Barros [40] stated that the disintegration of coated pectin beads under simulated intestinal fluids, and following viable probiotic cell release, occurred after one-hour exposure. As described in Pérez-Luna and González-Reynoso [41] the enhanced survival rates of encapsulated cells could be ascribed to the fact that the hydrogel networks could reduce the diffusion rate of acid inside the microparticle and that when the pH is changed to that found in the intestine swelling of the hydrogel capsules occur following cell release.

Incorporation of probiotics, prebiotics and synbiotics in foods at an industrial scale encompasses several microbiological, technological and economical challenges. Regarding the technological applications, much attention is dedicated to delivery strategies that are able to increase their chemical stability and viability during shelf life. For non-dairy products, application of probiotic cultures represents a great challenge, some of the alternatives used to assure cell viability while maintaining good sensory attributes are microencapsulation, assessment of novel sources of prebiotic ingredients and engineering of edible coatings for probiotic bacteria entrapment and delivery [42]. The advantage of encapsulation is that it constitutes an approach that can be applied in a number of foods to achieve a wide variety of functional features. In this context the incorporation of inulin in extruded beads containing *L. rhamnosus* GG, has been found beneficial for cells' survival in a probiotic apple juice during storage (90 days at 4 °C or 25 °C) providing acceptable sensory characteristics [43]. In good agreement with this result, studies made by Valero-Cases and Frutos [44] using Artichoke inulin showed that during long storage periods fermented vegetable juices with 2% of inulin enhanced *L. plantarum* survival after *in vitro* digestion. Similarly, encapsulation with pectin also improved the viability of *L. rhamnosus* in gastric environments at very low pH [45], while Gebara, Chaves [46] reported a similar study in which pectin encapsulated *Lactobacillus acidophilus* exhibited lesser reduction (1.51 log cycles) than non-encapsulated cells (3.54 log cycles) on incubation in gastric and intestinal juices. Reinforcing this, Li, Zhang [38]

demonstrated that *Lactobacillus* GG encapsulated in pectin hydrogel beads exhibited longer shelf life and higher potential for prevention of intestinal inflammation providing information for understanding the beneficial effects of encapsulation on clinical efficacy of probiotics.

However, the development of new preparation methods that can undergo an economically feasible transition from the lab-bench scale to industrial-scale production are needed. One good example of scaling up from lab scale into commercial products are Perkii beverages, containing microencapsulated *Lactobacillus casei* through alginate microgels with a core application centered in gastric protection, controlled intestinal release and enhanced probiotic viability, among others. Similarly, the present work undergoes a relatively simple technique using only safe and non-toxic food ingredients such as pectin which is commonly used in many food products. Exploitation of food-grade polymers, such as pectin, is one example of a future technology that has the potential to meet the challenge of broadening the range of food types into which probiotic ingredients can be successfully incorporated while looking for alternatives sources such as pectins from apple pomace helping to reassess its value and reduce the high amounts produced worldwide, disposal that represents a serious environmental and economic problem for industries [47].

4. Conclusions

Cell microencapsulation using effective, food-grade, economical, nontoxic, and easy to use coating materials such as pectin constitutes a real possibility to generalize the use of encapsulated probiotics in the food processing industries, especially the ones associated with adverse matrices such as fruit juices with low pHs. The two probiotic strains used in this study, *Lactobacillus casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 53103, widely recognized and used in the food industry, encapsulated with pectin and supplemented with inulin presented good survival profiles to storage conditions to a relatively extended shelf lifetime such as 6 weeks (42 days). Even though results after gastrointestinal challenges were not as high as expected, future assays will focus on optimizing the survival. All in all, it can be concluded that the time at which strains are harvested influence their performance and that older cultures of *L. casei* 393 and *L. rhamnosus* 53103 encapsulated in synbiotic pectin hydrogels with JAI present a promising future not only to what refers to its storage survival but also to exposure to harsh conditions as the ones imposed by the GI tract.

CRediT authorship contribution statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis, first draft of the manuscript, conceptualization, formal analysis and investigation, writing - original draft preparation, writing - review and editing were performed by Dr. María Clara Tarifa, Dr. Lorena Brugnoni, Dr. Cristian Piqueras and Dr. Diego Genovese.

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All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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