Food Research International 64 (2014) 560–566

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Edible methylcellulose-based films containing fructo-oligosaccharides as vehicles for lactic acid bacteria

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ARTICLE INFO

Article history:
Received 4 May 2014
Accepted 20 July 2014
Available online 1 August 2014

Keywords:
Lactobacillus
Methylcellulose films
Fructo-oligosaccharides
Protective compound
Plasticizer
Bacteria immobilization

ABSTRACT

The goal of this work was to investigate the physicochemical properties of methylcellulose (MC) based films as stabilizers of two strains of lactobacilli: *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 and *Lactobacillus plantarum* CIDCA 83114. The incorporation of 3% w/v fructo-oligosaccharides (FOS) into the MC film formulation improved the viability of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 after film preparation. *L. plantarum* CIDCA 83114 was intrinsically more resistant as no viability loss was observed upon preparation of the films in the absence of FOS.

Scanning electronic microscopy images also showed a good incorporation of microorganisms without affecting the homogeneity of the films. FTIR spectroscopy provided structural information about the bacteria-loaded films. Water sorption isotherms showed an impervious behavior at low a_w but on exceeding 0.7 of a_w the film started to dissolve and form syrup, causing a drastic drop of bacterial viability ($\log N/N_0 \leq -5$). Dynamic mechanical analysis (DMA) demonstrated that the incorporation of microorganisms into the MC films had no effect on vitreous transition temperatures. FOS incorporated into the MC films had a plasticizing effect.

Microorganism-loaded films were stored at relative humidities (RH) ranging from 11 to 75%. Both strains could be stored at 11% RH for 90 days. At 33 and 44% RH *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 could be stored up to 15 days and *L. plantarum* CIDCA 83114 up to 45 days. At 75% RH only *L. plantarum* CIDCA 83114 could be equilibrated ($\log N/N_0$: -2.05 ± 0.25), but CFU/g films were undetectable after 15 days of storage.

The results obtained in this work support the use of MC films containing FOS as a good strategy to immobilize lactic acid bacteria, with potential applications in the development of functional foods.

1. Introduction

The importance of lactic acid bacteria (LAB) and probiotics in the food and pharmaceutical industries highlights the need of preservation strategies ensuring an adequate viability in the final product. Hence, the incorporation of these microorganisms into edible films appears as a suitable approach to increase their survival upon dehydration and storage. Besides the small volumes occupied by the films, this strategy represents an effective way to increase cell density and protect them during storage and processing. All these advantages are especially relevant in the development of industrial applications.

Probiotic-loaded films have been frequently used as food coatings to take advantage of the microbial beneficial effects and extend the shelf-life of different products (Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010; López de Lacey, López-Caballero, Gómez-Estaca, Gómez-Guillén, & Montero, 2012; Moayednia et al., 2009; Tapia et al., 2007). In this regard, Sánchez-González, Quintero Saavedra, & Chiralt (2013) developed bioactive polymeric films as vehicles of bacteriocins produced in situ by a *Lactobacillus plantarum* strain. The antimicrobial activity of *Lactobacillus sakei* immobilized in sodium–caseinate films was also addressed (Gialamas et al., 2010). In addition, alginate films have proved to be adequate carriers for *L. plantarum* release, demonstrating good perspectives for pharmaceutical applications (Brachkova, Duarte, & Pinto, 2009, 2012, Brachkova et al., 2011).

Methylcellulyose (MC) is a low cost, edible and clear viscous polymer in aqueous environments (Li et al., 2002). Its unique properties as film forming agent led to several pharmaceutical and food applications (Bodvik et al., 2010; Lin, Wang, Wei, & Li, 2007; Pérez et al., 2008). However, to our knowledge, no reports on LAB-loaded MC films have been published hereto.

Abbreviations: LAB, lactic acid bacteria; MC, methylcellulose; FOS, fructo-oligosaccharides; FTIR, Fourier transform infrared; DMA, dynamic mechanical analysis; MRS, de Man, Rogosa, Sharpe broth; CFU, cell forming units; RH, relative humidity; ds, dried sample; a_w, water activity; T_g, glass transition temperature; T_m, gel–liquid crystal transition temperature.

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http://dx.doi.org/10.1016/j.foodres.2014.07.018
0963-9969/

Considering that MC film formulation involves heating and drying processes, sensitive lactic acid bacteria, like *Lactobacillus delbrueckii* subsp. *bulgaricus*, may be injured or dead. For this reason, the incorporation of protective compounds in the MC film formulation can be an adequate strategy to overcome this problem. The role of fructo-oligosaccharides (FOS) as bacterial protectants in processes involving dehydration has been reported in the last years (Chaluvadi et al., 2012; Golowczyc, Gerez et al., 2011; Schwab, Vogel, & Gänzle, 2007). Taking into account the prebiotic properties of FOS, their incorporation into MC films may have a double advantage: protection and prebiotic effect (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004).

The structural and mechanical properties of MC films have been recently addressed (Tavera-Quiroz, Lecot, Bertola, & Pinotti, 2013) and FOS-based edible films have also been developed (Ramesh & Siddalingaiya, 2006). However, the incorporation of FOS into MC films results in novel films whose physical–chemical properties deserve a careful analysis. Moreover, when attempting to include microorganisms in MC films, certain parameters like temperature and time of dehydration must be thoroughly controlled in order to avoid losses of viability. This requires a careful investigation of the bacteria-loaded MC films from both the physical–chemical and the microbiological points of view.

In this work, MC films were loaded with two strains of lactobacilli: *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 and *L. plantarum* CIDCA 83114. *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 is a highly sensitive strain upon any preservation process (Tymczyszyn et al., 2012) and *L. plantarum* CIDCA 83114 inhibits the growth and/or the activity of *Escherichia coli* O157:H7, *Shigella* and *Salmonella* (Golowczyc, Silva, Teixeira, De Antoni, & Abraham, 2011; Hugo, Kakisu, De Antoni, & Pérez, 2008; Kakisu, Abraham, Tironi Farinati, Ibarra, & De Antoni, 2013; Kakisu, Bolla, Abraham, de Urraza, & De Antoni, 2013). The effect of FOS included in the MC film on both bacterial viability and physicochemical properties of the films was investigated.

2. Materials and methods

2.1. Materials

MC (A4M, Methocel) (Dow, MI, USA), sorbitol (Merck, USA), FOS (Orafti Beneo p95, Germany), LiCl, $MgCl_2$, K_2CO_3, NaCl, KCl and K_2SO_4 (Anedra, Argentina) were used.

2.2. Methods

2.2.1. Film forming solution

To prepare the hydrocolloid solution, 1.5 g MC were slowly dispersed in 50 mL of distilled water at 80 °C, under constant stirring for 1 h. Once a homogeneous system was obtained, a total volume of 100 mL was made up with cold distilled water and the solution was kept under stirring until it attained room temperature. Afterwards, sorbitol was added as a plasticizer to a final concentration of 0.25% w/v, as determined in a previous work (Tavera-Quiroz et al., 2013). FOS, composed of oligosaccharides of different degree of polymerization (from 2 to 8), were also added to the hydrocolloid solution, to attain concentrations of 0 (control), 1, 2, 3 and 5% w/v. The obtained solution was sterilized using 0.2 μm sterile filters. These experiments allowed selecting the most suitable concentration of FOS for subsequent experiments (Sections 2.2.7 to 2.2.10).

2.2.2. Bacterial strains and growth conditions

L. delbrueckii subsp. *bulgaricus* CIDCA 333 and *L. plantarum* CIDCA 83114 were isolated from fermented milks (Garrote, Abraham & De Antoni, 2001; Gómez-Zavaglia, Abraham, Giorgieri, & De Antoni, 1999). The strains were maintained frozen at −80 °C in 120 g/L non-fat milk solids. Cultures were grown in MRS broth (de Man, Rogosa, & Sharpe, 1960) at 37 °C (*L. delbrueckii* subsp. *bulgaricus* CIDCA 333) and 30 °C (*L. plantarum* CIDCA 83114) in aerobic conditions.

100 mL of cultures in the stationary phase [grown overnight in MRS to attain approximately 5×10^8 CFU/mL for *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 (Tymczyszyn et al., 2012) and 1×10^{10} CFU/mL for *L. plantarum* CIDCA 83114 (Golowczyc, Silva, et al., 2011)] was harvested by centrifugation at 7000 ×*g* for 10 min and washed twice with 0.85% w/v NaCl.

2.2.3. Film preparation

Bacterial pellets were diluted into 17–25 mL of the film forming solution to obtain 1.1×10^{10} CFU/mL of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 and 2.7×10^{12} CFU/mL of *L. plantarum* CIDCA 83114. An aliquot of 1.5 mL of the resulting suspension was spread onto Petri dishes and dried in a forced air oven at 40 °C for 2.5 h. The obtained films (surface: 28.3 cm^2 and weight: 50 mg) were removed from the dishes and thickness was determined using a coating thickness gauge (Check Line DCN-900, New York, USA) for non-conductive materials on non-ferrous substrates.

2.2.4. Bacterial plate counts

50 mg of the bacteria-loaded films, corresponding to 1.5 mL of film forming solution, were re-hydrated in 5 mL of 0.85% w/v NaCl for 10 min to allow a complete hydration. The obtained suspensions were serially diluted and plated on MRS agar. Plates containing *L. plantarum* CIDCA 83114 were incubated at 30 °C for 24 h in aerobic conditions (Golowczyc, Silva, et al., 2011), and plates containing *L. delbrueckii* subsp. *bulgaricus* CIDCA 333, at 37 °C for 48 h in aerobic conditions (Tymczyszyn et al., 2012). This process was carried out any time that bacterial viability was determined [immediately after drying and during storage (see Section 2.2.10)].

2.2.5. Scanning electron microscopy

The bacteria-loaded films were frozen in liquid nitrogen and fractured using a cold scalpel blade. The samples were examined with a FEI model Quanta 200 electron microscope (The Netherlands). Samples were mounted onto bronze stubs by using a double-sided tape and examined without any metal or carbon coating at low pressure and an acceleration voltage of 12.5 kV.

2.2.6. FTIR spectra of the films with bacteria

FTIR spectra of the MC films were recorded in a transmission mode, on a Thermo Nicolet iS10 spectrometer (Thermo Scientific, MA, USA). The FTIR spectra were obtained in the 4000–400 cm^{-1} range, by co-adding 64 scans with 4 cm^{-1} spectral resolution (Tavera-Quiroz et al., 2013).

2.2.7. Water sorption isotherm

50 mg of the bacteria-loaded films (28.3 cm^2) were equilibrated at 4 °C in atmospheres of the following saturated salts: LiCl, $MgCl_2$, K_2CO_3, NaCl, KCl and K_2SO_4 giving relative humidities (RH) of 11, 33, 44, 75, 85 and 97%, respectively.

Moisture contents of the bacteria-loaded films were determined by measuring their weight loss, upon drying in a vacuum oven at 105 °C until constant weight (AOAC 1980). Moisture results were expressed as grams of water per 100 g of dried sample (ds).

GAB (Guggenheim-Anderson-de Boer) and Iglesias–Chirife models were used to fit sorption isotherm data. GAB isotherm model can be expressed as follows:

$$M_w = \frac{m_0 C K_{aw}(1 - K_{aw} + C K_{aw})}{(1 - K_{aw})} \quad (1)$$

where M_w is the equilibrium moisture content at a given water activity (a_w), m_0 is the monolayer value (g water/g solids) and C and K_{aw} are the GAB constants (Galdeano, Mali, Grossmann, Yamashita, & García, 2009).

The model developed by Iglesias and Chirife (1981) is expressed as follows:

$$M_w = \frac{Aa_w}{(1-a_w)} + B \quad (2)$$

where M_w is the moisture content at a given a_w; A and B are constants. This model was found to be suitable for products with high sugar content. The values of m_0, C, K_{aw}, A and B in Eqs. (1) and (2) are shown in Table 1.

2.2.8. Water vapor barrier properties

Water vapor permeability (WVP, g m^{-1} s^{-1} Pa^{-1}) tests were performed using a modified ASTM method E96 as described previously (Tavera-Quiroz et al., 2013). In brief, each sample was sealed over a permeation cell which was maintained at 20 °C. A driving force of 1753.55 Pa, corresponding to 75% RH gradient across the film, was used. After steady state conditions were reached, the permeation cells were weighed (0.0001 g) every hour up to a total of 8 h.

2.2.9. Dynamic mechanical analysis (DMA)

DMA assays were conducted in dynamic–mechanical thermal equipment Q800 (TA Instruments, New Castle, DE, USA) using a tension clamp with a liquid N_2 cooling system. Film probes with a rectangular geometry (30 mm length, 6 mm width and thickness values between 40 and 50 μm) were assayed. Amplitude sweeps from 1 to 50 μm at a fixed frequency (5 Hz) were performed. Multi-frequency sweeps (1, 5, 10 and 15 Hz) at a fixed amplitude from −100 to 250 °C at 5 °C/min were carried out, with an isotherm of 10 min at −100 °C. Storage (E′), loss (E″) modulus and tan δ (E″/E′) curves as a function of temperature were recorded and analyzed using the software Universal Analysis 2000 (New Castle, DE, USA). Temperatures of the relaxation processes were determined through the peaks in tan δ curves (Rivero, García, & Pinotti, 2012).

2.2.10. Storage

The bacteria-loaded films were equilibrated for 15 days at different RH (Section 2.2.7.), and stored for 90 days at 4 °C. Viability was determined every 15 days, as described in Section 2.2.4.

2.2.11. Reproducibility of results

All experiments were performed on duplicate samples using three independent cultures of bacteria. The relative differences were reproducible irrespective of the cultures used. Differences were tested with paired sample t tests, and if $P < 0.05$ the difference was considered statistically significant.

3. Results and discussion

3.1. Effect of FOS as protective compounds

The preparation of bacteria-loaded films includes two steps potentially harmful for microorganisms: the osmotic effect of the film forming solution onto the loaded bacteria and the dehydration effect of drying in a forced air oven. The effect of FOS on bacterial viability was analyzed in both steps, by including different concentrations of these oligosaccharides in the film forming solution. Loading both strains into the film forming solution did not lead to a significant decrease of viability even in the absence of FOS (data not shown). However, the dehydration step led to a significant decrease in the viability of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 when no FOS were included in the films (log $N/N_0 = -3.18 \pm 0.33$) (Fig. 1A). Increasing the concentration of FOS in the films had a clear protective effect up to 3% w/v FOS and no further improvement was observed at higher concentrations (5% w/v) (Fig. 1A). *L. plantarum* CIDCA 83114 showed a greater intrinsic resistance toward dehydration processes as the decrease of viability in MC films prepared without FOS was log $N/N_0 = -1.45 \pm 0.38$ (Fig. 1B). The addition of FOS up to 5% w/v did not significantly increase *L. plantarum* CIDCA 83114 viability.

The protective effect of sugars in processes involving bacterial dehydration (i.e.: freeze-drying, spray drying, freezing, etc.) is well-known. FOS have been reported as good protectants in freeze-drying and spray drying of lactobacilli (Chaluvadi et al., 2012; Golowczyc, Gerez, et al., 2011; Schwab et al., 2007). Hincha, Zuther, and Heyer (2003) reported that FOS of different degrees of polymerization prevent fusion and leakage of egg phosphatidylcholine lipid membranes in the dried state. The interaction of FOS with phosphate groups decreases the phase transition temperatures (T_m) of lipid membranes. According to the water replacement theory of membrane protection, the interaction of sugars with the polar heads of lipid membranes in the dried state leads to a decrease of T_m. Therefore, membranes dehydrated in the presence of sugars remain in the liquid crystalline phase as if they were hydrated (Crowe, Carpenter, & Crowe, 1998; Crowe, Hoekstra, & Crowe, 1992). This mechanism supports the protective effect of FOS observed for *L. delbrueckii* subsp. *bulgaricus*.

Table 1
Parameters of GAB and Iglesias and Chirife equations (Eqs. (1) and (2) in the text).

GAB model parameters (Eq. (1))			
m_o	C	K_{aw}	R^2
12.56	−56.53	0.908	0.992
Iglesias–Chirife's model parameters (Eq. (2))			
A	B		R^2
2.17	17.98		0.991

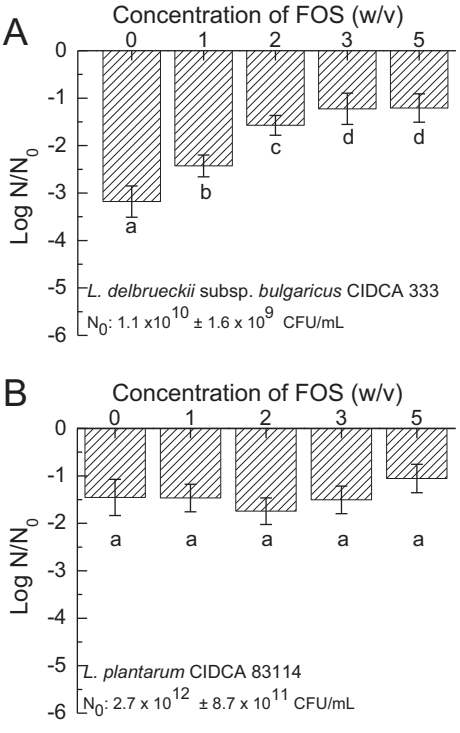

Fig. 1. Log N/N_0 of microorganisms-loaded into MC films containing different concentrations of FOS. A: *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333; B: *L. plantarum* CIDCA 83114. N_0 corresponds to CFU/mL of film forming solutions (before drying) and was $1.1 \times 10^{10} \pm 1.6 \times 10^9$ CFU/mL for *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 and $2.7 \times 10^{12} \pm 8.7 \times 10^{11}$ CFU/mL for *L. plantarum* CIDCA 83114. Experiments were repeated twice (n = 2). Different letters indicate significant differences ($P < 0.05$).

It was reported that the T_m of dehydrated *L. plantarum* strains is 20 °C (Linders, Wolkers, Hoekstra, & van 't Riet, 1997) and the T_m of *L. delbrueckii* subsp. *bulgaricus*, 35–40 °C (Oldenhof, Wolkers, Fonseca, Passot, & Marin, 2005). This supports the greater intrinsic resistance of *L. plantarum* CIDCA 83114 to dehydration.

Santivaragkna, Naumann, Kulozik, and Foerst (2010) reported that sorbitol protects lactobacilli during drying by depressing the T_m of their lipids. The interaction of sorbitol with lactobacilli lipid membranes occurs through phosphate groups via the formation of hydrogen-bonds (Santivaragkna et al., 2010). The presence of 0.25% w/v sorbitol in *L. plantarum* CIDCA 83114-loaded films did not have a protectant effect as no significant differences were observed between films containing or not containing FOS (0% FOS) (Fig. 1B). The absence of FOS (0% FOS) in the *L. delbrueckii* subsp. *bulgaricus* CIDCA 333-loaded films led to a significant drop of viability ($P < 0.05$) (Fig. 1A). Taking into account that the viability of this strain only increased after increasing the concentration of FOS in the films, it can be concluded that the presence of 0.25% w/v sorbitol does not have relevance in terms of bacterial protection in the present experimental conditions. This protection is given by the presence of FOS.

3.2. Structural analysis of the films

Fig. 2 shows the scanning microscopy images of both bacterial strains completely embedded in the film matrix. The homogeneity of the films indicates their structural integrity, which was not affected by the presence of microorganisms. On the other hand, the bacterial morphology was not altered upon embedding in the MC films.

Fig. 3 depicts the FTIR spectra of MC films, loaded or not with microorganisms. A strong band at 3380 cm^{-1} was observed in all the three spectra. This band can be ascribed to the νOH vibrational mode of all compounds having OH groups (MC, sorbitol, FOS). OH groups of bacteria polysaccharides also contribute to the absorption of this band. As all spectra were registered immediately after preparation of the films, the contribution of water in this region is scarce. In the 1750–1540 cm^{-1} spectral region, two weak bands were observed in the spectrum of MC films without bacteria (Fig. 3A). These bands can be attributed to small amounts of water trapped in the film. In the same region, both bacteria-loaded films showed two stronger bands, at 1651 and 1547 cm^{-1}, corresponding to amide I and II bands of proteins (Fig. 3B and C) (Gerbino, Mobili, Tymczyszyn, Fausto, & Gómez-Zavaglia, 2011). MC films strongly absorbed in the 1200–900 cm^{-1} region because of the contribution of the C–O–C glycosidic linkage (particularly those of MC and FOS) (Fig. 3A) (Kizil, Irudayaraj, & Seetharaman, 2002). Moreover, P–O–P bonds of DNA also absorbed in this region (Beekes, Lasch, & Naumann, 2007). Therefore, these vibrational modes also contributed to this band in the bacteria-loaded films (Fig. 3B and C).

3.3. Physical–chemical properties of the films

Considering the films as matrices carrying viable microorganisms, their physical–chemical properties should be analyzed simultaneously considering: a) the generation of an adequate environment that assures bacterial viability and b) the design of films with adequate plasticization properties to avoid brittleness in storage conditions.

As films are hygroscopic materials and high water contents are detrimental for bacterial preservation, the control of moisture content during processing and storage is crucial. In this respect, moisture sorption isotherms provide information to predict changes in the stability of biodegradable materials and to assess the maximum moisture compatible with bacterial viability. This information will determine the most appropriate storage conditions.

Fig. 4A and B shows that the higher the water activity, the higher the equilibrium moisture content. Adsorption isotherms were type III (J-type) isotherms, characteristic of materials with high sugar content

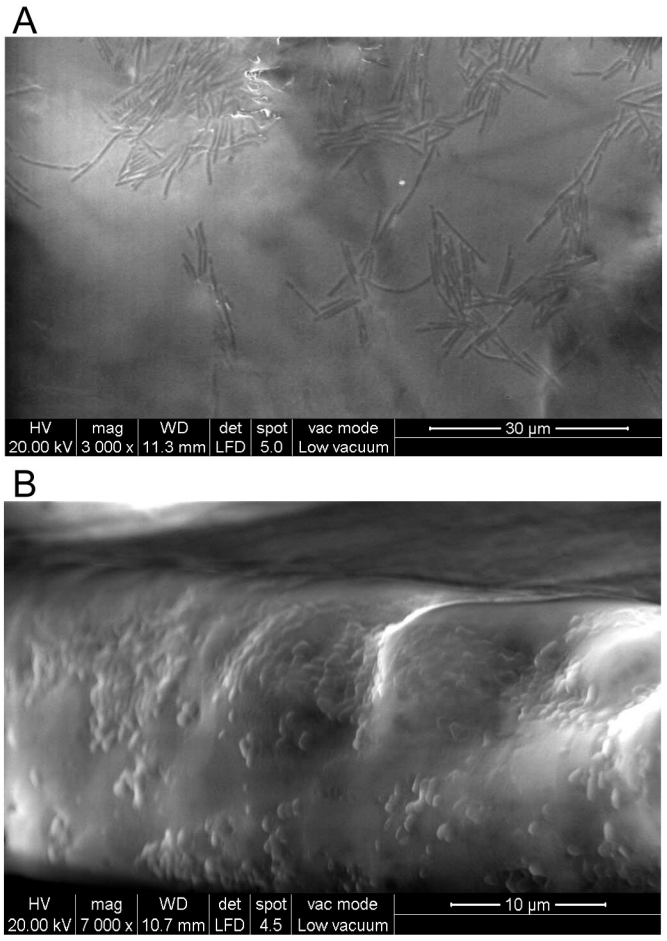

Fig. 2. Scanning electron microscopy of bacteria-loaded films: A: *L. delbrueckii* subsp. *bulgaricus* CIDCA 333; B: *L. plantarum* CIDCA 83114. Scale bars are indicated in the images.

(Falade, Olukini, & Adegoke, 2004). This is in accordance with the classification of Labuza, McNally, Gallagher, Hawkes, & Hurtado (1972). Both isotherms showed a crystalline pattern which, in regions of

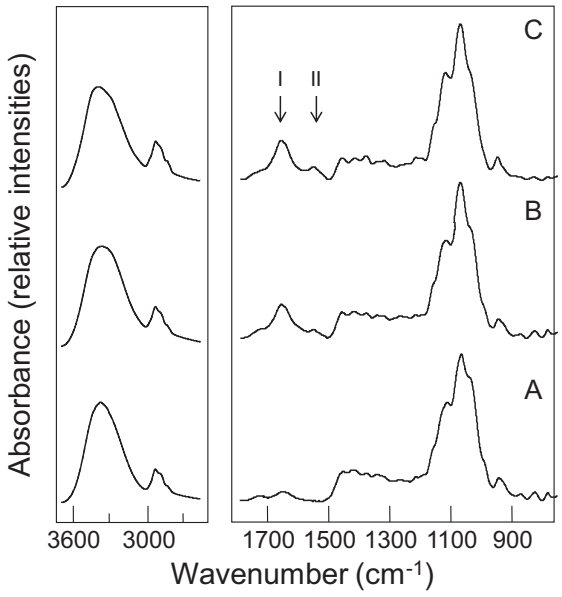

Fig. 3. FTIR spectra of: A: MC films; B: *L. delbrueckii* subsp. *bulgaricus* CIDCA 333-loaded films; C: *L. plantarum* ÇIDCA 83114-loaded films. Spectra were registered immediately after preparation of the films. I and II denote amide I and II bands.

low a_w, showed nearly impervious behavior, but on exceeding 0.7 of a_w the film started to dissolve and form syrup. This behavior corresponds to that of some products containing relatively high amounts of sugars besides the other polymer constituents (Iglesias & Chirife, 1981). The formation of syrup in this work was concomitant with a drastic drop in bacterial viability (Fig. 4A and B). It is known that bacterial viability is higher in low a_w environments. In this regard, the viability of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 freeze-dried in the presence of galacto-oligosaccharides is strongly determined by a_w. Indeed, bacterial viability does not decrease after 45 days of storage at 4 °C when a_w is lower than 0.33 (Tymczyszyn et al., 2012). Moreover, low molecular mobility also improves bacterial stability, and this condition is obtained at low water contents and low storage temperatures (Tymczyszyn et al., 2012).

MC-based films were tested by DMA in order to study the effect of the different components on the thermal properties. Immediately after the drying process, the tan δ curve for MC films (full line in Fig. 5) showed two relaxations, β and α (Fig. 5). The β relaxation at around −21 °C is characteristic of the local motion of side groups in polysaccharides. Other authors considered this β relaxation in hydrophilic materials as a typical water relaxation, as a consequence of hydroxyl motions, favored by water molecules (Fernandez-Carretero et al., 2010; Mucha & Pawlak, 2005). The second peak corresponds to α relaxation and the temperature at which it takes place can be labeled as dynamic glass transition temperature (T_g). This transition is related to relaxation phenomena due to mobility activation processes of noncrystalline local regions, macromolecular backbone, specific macromolecular parts or macromolecular chain terminal groups (Samios, Tokumoto, & Denardin, 2006).

The incorporation of microorganisms into MC film (dashed lines in Fig. 5) had no effect on the location of both β relaxation and T_g. The addition of FOS (dash dot lines in Fig. 5) interfered with the hydrogen bonds among the hydroxyl groups of the polymer molecules, hence T_g shifted to a lower value, indicating the plasticizer effect of FOS. This tendency was more evident in the presence of FOS and bacteria simultaneously (dash double dot line in Fig. 5). These findings may be explained in relation to the moisture content (Fig. 4). The β relaxation underwent a displacement toward higher temperatures and also a better peak definition related to higher water content (Fig. 5). According to Park and Ruckenstein (2001) the shift of the T_g would prove the compatibility between both FOS and MC as well as the plasticizing effect of FOS.

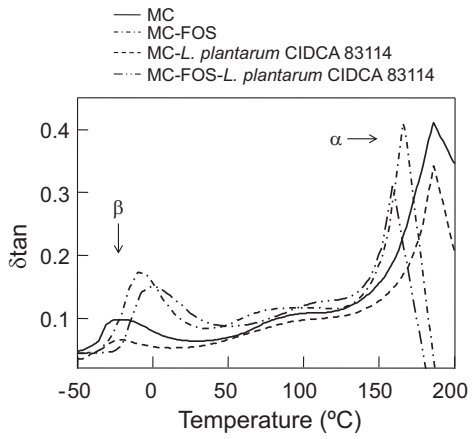

Fig. 5. Effect of the incorporation of FOS and/or bacteria on the dynamic mechanical spectra of MC films. Full lines, MC; dash dot lines MC–FOS; dash lines, MC-*L. plantarum* CIDCA 83114; dash double dot lines, MC–FOS-*L. plantarum* CIDCA 83114.

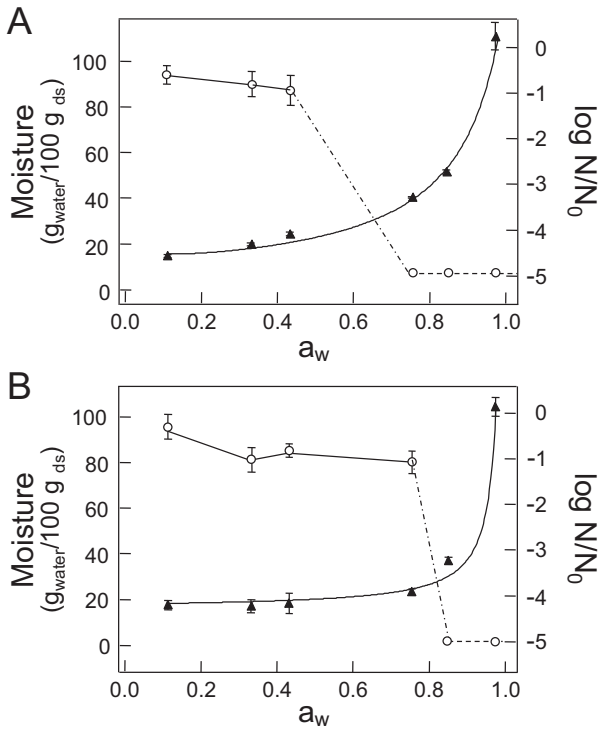

Fig. 4. Moisture content at 4 °C (▲) and log N/N_0 (○) as a function of a_w. A: *L. delbrueckii* subsp. *bulgaricus* CIDCA 333; B: *L. plantarum* CIDCA 83114. N corresponds to CFU/g film at each a_w and N_0, to CFU/g film immediately after dehydration in forced air oven at 40 °C. Dash lines indicate that log $N/N_0 \leq -5$ (corresponding to the lowest dilution made to plate count). Dash dot lines just connect two consecutive experimental points where log N/N_0 decreases from one to the other. This line is uncertain because no points in between were analyzed.

3.4. Storage of the bacteria-loaded films

Considering that FOS are good bacterial protectants but in high concentrations worsen the quality of MC films, the concentration of FOS to be used in storage experiments was selected making a balance between protective effect and quality of the film. Therefore, *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 was loaded into films containing 3% w/v FOS and *L. plantarum* CIDCA 83114, into films containing 1% w/v FOS (see Fig. 1). Even when 1% w/v FOS did not have beneficial effects on the survival of the latter strain, they may act as prebiotics, thus giving an added value for potential food applications.

Fig. 6 shows the evolution of log N/N_0 during storage at 4 °C after equilibration at different RH. *L. plantarum* CIDCA 83114 showed a better performance upon storage at 11, 33 and 44% RH (Fig. 6B). At 33 and 44% RH, *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 could be stored up to 15 days (Fig. 6A). At 75% RH, it was undetectable after equilibration. *L. plantarum* CIDCA 83114 could be equilibrated at 75% RH (log N/N_0: -2.05 ± 0.25), but it was undetectable after 15 days of storage.

The lineal regression of the data plotted in Fig. 6 allowed describing bacterial inactivation rate in each storage condition according to Eq. (3):

$$\text{Log } N/N_0 = -kt \quad (3)$$

where N is the CFU/g film at a given time of storage, N_0 is the CFU/g film immediately after dehydration in forced air oven at 40 °C ($2.9 \times 10^{11} \pm 5.0 \times 10^{10}$ CFU/g for *L. plantarum* CIDCA 83114 and $4.0 \times 10^{8} \pm 1.0 \times 10^{7}$ CFU/g for *L. delbrueckii* subsp. *bulgaricus* CIDCA 333), t is the time of storage expressed in days and k is the constant of bacterial inactivation (expressed in $days^{-1}$) (Table 2).

Considering that benefits of probiotic consumption can be obtained when the food contains at least 6–7 log CFU of viable microorganisms per gram of product at the end of shelf-life of commercial products (Aquilina et al., 2013; Hill et al., 2014; Phuapaiboon, Leenanon, & Levin, 2013; Tripathi & Giri, 2014), k values represent an important

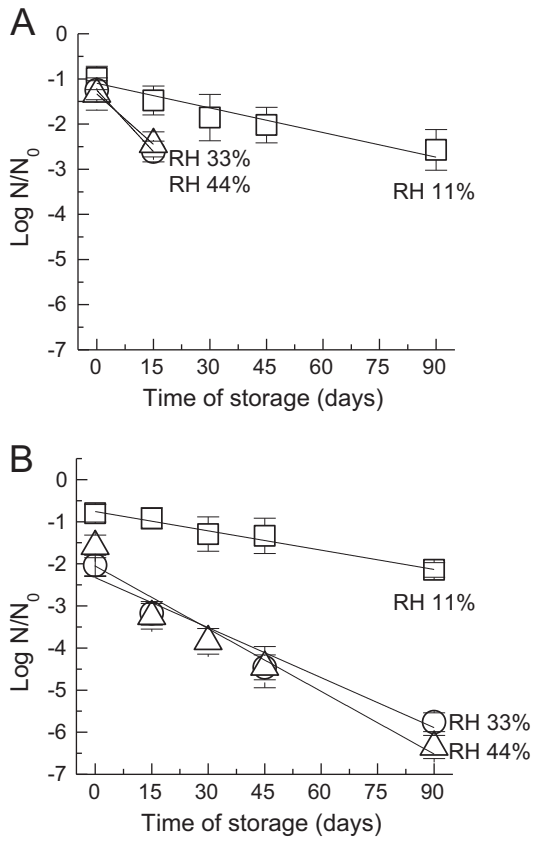

Fig. 6. Log N/N_0 of microorganisms-loaded in MC films and stored 90 days at 4 °C at different RH: 11% (□), 33% (○), 44% (Δ). Solid lines show linear regression for each condition. A: *L. delbrueckii* subsp. *bulgaricus* CIDCA 333-loaded in MC films containing 3% FOS w/v; B: *L. plantarum* CIDCA 83114-loaded in MC films containing 1% FOS w/v; N indicates CFU/g film in each storage condition and N_0, CFU/g film immediately after dehydration in forced air oven at 40 °C. N_0 was $2.9 \times 10^{11} \pm 5.0 \times 10^{10}$ CFU/g for *L. plantarum* CIDCA 83114 and $4.0 \times 10^{8} \pm 1.0 \times 10^{7}$ CFU/g for *L. delbrueckii* subsp. *bulgaricus* CIDCA 333.

tool to determine the shelf-life of a given product at the storage conditions (temperature and RH).

The results obtained indicate that *L. plantarum* CIDCA 83114 can be stored for longer periods at higher RH than *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 (note that the inactivation constant k of *L. plantarum* CIDCA 83114 was lower at all the RH) (Table 2). This behavior defines potential applications of the bacteria-loaded films. The MC films prepared in this work are light, low cost, do not occupy great volumes and have a low permeability to water vapor ($3.5–6.1 \times 10^{-11}$ g m^{-1} s^{-1} Pa^{-1} as determined in this work). Moreover, the preparation of the bacteria-loaded films only takes 2.5 h. Therefore, loading microorganisms in MC films containing FOS appears as a good strategy to preserve and transport lactic acid bacteria, including those that are highly sensitive to dehydration and storage (i.e.: *L. delbrueckii* subsp. *bulgaricus*). The greater capacity of *L. plantarum* CIDCA 83114 to survive in environments with higher RH, together with its inhibitory properties against *E. coli* O157:H7 and *Shigella*

Table 2
Inactivation constants of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333-loaded in MC films containing 3% FOS w/v and *L. plantarum* CIDCA 83114-loaded in MC films containing 1% FOS w/v at different RH.

RH	*L. delbrueckii* subsp. *bulgaricus* CIDCA 333	*L. plantarum* CIDCA 83114
11	0.0181 R^2: 0.922	0.0152 R^2: 0.990
33	0.0904 [a]	0.0395 R^2: 0.955
44	0.0742 [a]	0.0496 R^2: 0.944

[a] Only two points were considered because after 15 days of storage, cultivability dropped to undetectable values.

(Golowczyc, Silva, et al., 2011; Hugo et al., 2008; Kakisu, Abraham, et al., 2013; Kakisu, Bolla, et al., 2013), supports the application of *L. plantarum* CIDCA 83114-loaded films in the formulation of functional foods.

4. Conclusion

The suitability of MC films containing FOS as vehicles of two strains of lactobacilli was analyzed in this work. The integrative approach including both the physicochemical characterization of the films and the stability of the loaded microorganisms provided a complete picture about the best conditions to immobilize lactobacilli.

The results obtained represent an easy and low cost strategy to immobilize and store probiotics in concentrations compatible with those required by international organisms like EFSA or FDA (Aquilina et al., 2013; Hill et al., 2014; Phuapaiboon et al., 2013; Tripathi & Giri, 2014). Besides the contribution to the preservation of lactic acid bacteria, this strategy supports potential applications of the immobilized microorganisms in the development of functional products containing both probiotics and prebiotics.

Acknowledgments

This work was supported by the Argentinean Agency for the Scientific and Technological Promotion (ANPCyT) (Projects PICT/2011/0226 and PICT/2012/0415) and the Argentinean National Research Council (CONICET) (PIP2012-2014114-201101-00024). Authors acknowledge J. Lecot for technical assistance. P.M., N.B., A.P. and A.G.-Z. are members of the research career CONICET. M.J.T.-Q. and N.R. are doctoral fellows from CONICET and ANPCyT, respectively.

Author's contributions

N.R., M.J.T.-Q, N.B. and P.M. did the experimental work. A.P. and A.G.-Z coordinated the work (analysis of results, discussion and writing of the manuscript). All authors have approved the final version of the manuscript.

References

Aquilina, G., Bach, A., Bampidis, V., Bastos, M. L., Flachowsky, G., & Gralak, M. A. (2013). Scientific opinion on the safety and efficacy of Probiotic LACTINA® (*Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Streptococcus thermophilus* and *Enterococcus faecium*) for chickens for fattening and piglets. *EFSA Journal*, *11*, 3170–3183.

Beekes, M., Lasch, P., & Naumann, D. (2007). Analytical applications of Fourier transform-infrared (FT-IR) spectroscopy in microbiology and prion research. *Veterinary Microbiology*, *123*, 305–319.

Bodvik, R., Dedinaite, A., Karlson, L., Bergström, M., Bäverbäck, P., Pedersen, J. S., Edwards, K., Karlsson, G., Varga, I., & Claesson, P. M. (2010). Aggregation and network formation of aqueous methylcellulose and hydroxypropylmethylcellulose solutions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *354*, 162–171.

Brachkova, M. I., Duarte, A., & Pinto, J. F. (2009). Evaluation of the viability of *Lactobacillus* spp. after the production of different solid dosage forms. *Journal of Pharmaceutical Sciences*, *98*, 3329–3339.

Brachkova, M., Duarte, A., & Pinto, J. (2012). Alginate films containing viable *Lactobacillus plantarum*: Preparation and *in vitro* evaluation. *AAPS PharmSciTech*, *13*, 357–363.

Brachkova, M. I., Marques, P., Rocha, J., Sepodes, B., Duarte, M. A., & Pinto, J. F. (2011). Alginate films containing *Lactobacillus plantarum* as wound dressing for prevention of burn infection. *The Journal of Hospital Infection*, *79*, 375–377.

Chaluvadi, S., Hotchkiss, A. T., Jr., Call, J. E., Luchansky, J. B., Phillips, J. G., Liu, L. S., & Yam, K. L. (2012). Protection of probiotic bacteria in a synbiotic matrix following aerobic storage at 4 °C. *Beneficial Microbes*, *3*, 175–187.

Crowe, J. H., Carpenter, J. F., & Crowe, L. M. (1998). The role of vitrification in anhydrobiosis. *Annual Review of Physiology*, *60*, 73–103.

Crowe, J. W., Hoekstra, F. A., & Crowe, L. M. (1992). Anydrobiosis. *Annual Review of Physiology*, *54*, 570–599.

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

Falade, K. O., Olukini, I., & Adegoke, G. O. (2004). Adsorption isotherm and heat of sorption of osmotically pretreated and air-dried pineapple slices. *European Food Research and Technology*, *218*, 540–543.

Fernandez-Carretero, F. J., Riande, E., del Río, C., Sanchez, F., Acosta, J. L., & Compan, V. (2010). Preparation and characterization of hybrid membranes based on Nafion®

using partially sulfonated inorganic fillers. *Journal of New Materials for Electrochemical Systems*, *13*, 83–93.
Galdeano, M. C., Mali, S., Grossmann, M. V. E., Yamashita, F., & García, M. A. (2009). Effects of plasticizers on the properties of oat starch films. *Materials Science and Engineering: C*, *29*, 532–538.
Garrote, G. L., Abraham, A. G., & De Antoni, G. L (2001). Chemical and microbiological characterisation of kefir grains. *Journal of Dairy Research*, *68*, 639–652.
Gerbino, E., Mobili, P., Tymczyszyn, E., Fausto, R., & Gómez-Zavaglia, A. (2011). FTIR spectroscopy structural analysis of the interaction between *Lactobacillus kefir* S-layers and metal ions. *Journal of Molecular Structure*, *987*, 186–192.
Gialamas, H., Zinoviadou, K. G., Biliaderis, C. G., & Koutsoumanis, K. P. (2010). Development of a novel bioactive packaging based on the incorporation of *Lactobacillus sakei* into sodium–caseinate films for controlling *Listeria monocytogenes* in foods. *Food Research International*, *43*, 2402–2408.
Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutrition Research Reviews*, *17*, 259–275.
Golowczyc, M., Gerez, C., Silva, J., Abraham, A., Antoni, G., & Teixeira, P. (2011). Survival of spray-dried *Lactobacillus kefir* is affected by different protectants and storage conditions. *Biotechnology Letters*, *33*, 681–686.
Golowczyc, M., Silva, J., Teixeira, P., De Antoni, G., & Abraham, A. (2011). Cellular injuries of spray-dried *Lactobacillus* spp. isolated from kefir and their impact on probiotic properties. *International Journal of Food Microbiology*, *144*, 556–560.
Gómez-Zavaglia, A., Abraham, A., Giorgieri, S., & De Antoni, G. (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.
Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Berni Canani, R., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014, June). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, http://dx.doi.org/10.1038/nrgastro.2014.66 (advance online publication).
Hincha, D. K., Zuther, E., & Heyer, A. G. (2003). The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1612*, 172–177.
Hugo, A. A., Kakisu, E., De Antoni, G. L., & Pérez, P. F. (2008). Lactobacilli antagonize biological effects of enterohaemorrhagic *Escherichia coli* in vitro. *Letters in Applied Microbiology*, *46*, 613–619.
Iglesias, H. A., & Chirife, J. (1981). An equation for fitting uncommon water sorption isotherms in foods. *Lebensmittel-Wissenschaft und Technolgie*, *14*, 105–106.
Kakisu, E., Abraham, A. G., Tironi Farinati, C., Ibarra, C., & De Antoni, G. L. (2013a). *Lactobacillus plantarum* isolated from kefir protects vero cells from cytotoxicity by type-II Shiga toxin from *Escherichia coli* O157:H7. *Journal of Dairy Research*, *80*, 64–71.
Kakisu, E., Bolla, P., Abraham, A. G., de Urraza, P., & De Antoni, G. L. (2013b). *Lactobacillus plantarum* isolated from kefir: Protection of cultured Hep-2 cells against *Shigella* invasion. *International Dairy Journal*, *33*, 22–26.
Kizil, R., Irudayaraj, J., & Seetharaman, K. (2002). Characterization of irradiated starches by using FT-Raman and FTIR spectroscopy. *Journal of Agricultural and Food Chemistry*, *50*, 3912–3918.
Labuza, T. P., McNally, L., Gallagher, D., Hawkes, J., & Hurtado, F. (1972). Stability of intermediate moisture foods. 1. Lipid oxidation. *Journal of Food Science*, *37*, 154–159.
Li, L., Shan, H., Yue, C. Y., Lam, Y. C., Tam, K. C., & Hu, X. (2002). Thermally induced association and dissociation of methylcellulose in aqueous solutions. *Langmuir*, *18*, 7291–7298.
Lin, S. -Y., Wang, S. -L., Wei, Y. -S., & Li, M. -J. (2007). Temperature effect on water desorption from methylcellulose films studied by thermal FT-IR microspectroscopy. *Surface Science*, *601*, 781–785.
Linders, L. J. M., Wolkers, W. F., Hoekstra, F. A., & van 't Riet, K. (1997). Effect of added carbohydrates on membrane phase behavior and survival of dried *Lactobacillus plantarum*. *Cryobiology*, *35*, 31–40.
López de Lacey, A. M., López-Caballero, M. E., Gómez-Estaca, J., Gómez-Guillén, M. C., & Montero, P. (2012). Functionality of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* incorporated to edible coatings and films. *Innovative Food Science & Emerging Technologies*, *16*, 277–282.
Moayednia, N., Ehsani, M. R., Emamdjomeh, Z., Asadi, M. M., Mizani, M., & Mazaheri, A. F. (2009). The effect of sodium alginate concentrations on viability of immobilized *Lactobacillus acidophilus* in fruit alginate coating during refrigerator storage. *Australian Journal of Basic and Applied Sciences*, *3*, 3213–3216.
Mucha, M., & Pawlak, A. (2005). Thermal analysis of chitosan and its blends. *Thermochimica Acta*, *427*, 69–76.
Oldenhof, H., Wolkers, W. F., Fonseca, F., Passot, S., & Marin, M. (2005). Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: An *in situ* Fourier transform infrared spectroscopy study. *Biotechnology Progress*, *21*, 885–892.
Park, J. S., & Ruckenstein, E. (2001). Viscoelastic properties of plasticized methylcellulose and chemically crosslinked methylcellulose. *Carbohydrate Polymers*, *46*, 373–381.
Pérez, O. E., Sanchez, C. C., Pilosof, A. M. R., & Patino, J. M. R. (2008). Dynamics of adsorption of hydroxypropyl methylcellulose at the air–water interface. *Food Hydrocolloids*, *22*, 387–402.
Phuapaiboon, P., Leenanon, B., & Levin, R. (2013). Effect of *Lactococcus lactis* immobilized within pineapple and yam bean segments, and Jerusalem artichoke powder on its viability and quality of yogurt. *Food and Bioprocess Technology*, *6*, 2751–2762.
Ramesh, M. N., & Siddalingaiya, G. P. (2006). Edible films and coatings based on fructooligosaccharides with probiotic properties. Patent Wipo WO/2006/103698.
Rivero, S., García, M. A., & Pinotti, A. (2012). Heat treatment to modify the structural and physical properties of chitosan-based films. *Journal of Agricultural and Food Chemistry*, *60*, 492–499.
Samios, D., Tokumoto, S., & Denardin, E. L. G. (2006). Investigation of the large plastic deformation of iPP induced by plane strain compression: Stress–strain behavior and thermo-mechanical properties. *International Journal of Plasticity*, *22*, 1924–1942.
Sánchez-González, L., Quintero Saavedra, J. I., & Chiralt, A. (2013). Physical properties and antilisterial activity of bioactive edible films containing *Lactobacillus plantarum*. *Food Hydrocolloids*, *33*, 92–98.
Santivaragkna, C., Naumann, D., Kulozik, U., & Foerst, P. (2010). Protective effects of sorbitol during the vacuum drying of *Lactobacillus helveticus*: An FT-IR study. *Annals of Microbiology*, *60*, 235–242.
Schwab, C., Vogel, R., & Gänzle, M. G. (2007). Influence of oligosaccharides on the viability and membrane properties of *Lactobacillus reuteri* TMW1.106 during freeze-drying. *Cryobiology*, *55*, 108–114.
Tapia, M. S., Rojas-Graü, M. A., Rodríguez, F. J., Ramírez, J., Carmona, A., & Martin-Belloso, O. (2007). Alginate- and gellan-based edible films for probiotic coatings on fresh-cut fruits. *Journal of Food Science*, *72*, E190–E196.
Tavera-Quiroz, M. J., Lecot, J., Bertola, N., & Pinotti, A. (2013). Stability of methylcellulose-based films after being subjected to different conservation and processing temperatures. *Materials Science and Engineering: C*, *33*, 2918–2925.
Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, *9*, 225–241.
Tymczyszyn, E. E., Sosa, N., Gerbino, E., Hugo, 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.