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Whey protein-kefiran films as driver of probiotics to the gut

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ABSTRACT

In this work, an alternative matrix for probiotics delivery constituted by whey proteins and polysaccharide kefiran was investigated. Edible transparent and homogeneous films containing the microorganisms *Lactobacillus paracasei* CIDCA 8339 and *Kluyveromyces marxianus* CIDCA 8154 with probiotic characteristics have been developed. Inclusion of microorganisms did not modify thickness, color or mechanical characteristics of films. This matrix showed good capability protecting the included probiotics from stress during film obtaining process and through gastrointestinal passage simulation. At the end of the applied gastrointestinal tract model, $7.1.10^5$ CFU/cm^2 *K. marxianus* CIDCA 8154 and $1.65.10^7$ CFU/cm^2 *L. paracasei* CIDCA 8339 remain viable, having decreased less than 0.6 logarithm cycle with respect to those originally included, even after 57 days of films storage.

1. Introduction

Consumer concerns regarding healthy diets and wellness have led to increase the consumption of foods containing probiotics and prebiotics. Probiotics are defined as live microorganisms which when administered in adequate amounts confer a benefit to the consumer's health (Hill et al., 2014). Prebiotics are non-digestible carbohydrates that reach the colon, where they are selectively fermented stimulating the growth and/or activity of one or a limited number of beneficial bacteria (Gibson & Roberfroid, 1995).

The most commonly used microorganisms in development of commercial probiotic products and in research belong to genera *Lactobacillus* and *Bifidobacterium* whereas those probiotic yeasts are represented mainly by *Saccharomyces spp*. (O'Toole, Marchesi, & Hill, 2017). Probiotics must resist food processing, storage and reach the small intestine viable overpassing injurious effects that take place during the passage through the gastrointestinal tract (Tripathi & Giri, 2014).

Probiotics and prebiotics, or their combinations, could be good alternatives in new food developments due to its ability to improve gut health by modulating microbiota (Tuohy, Probert, Smejkal, & Gibson, 2003). Probiotics incorporated into biopolymeric matrices allow development of active/bioactive food related materials with several applications for example in fruits, fish, bread and snacks as was recently summarized by Guimarães, Abrunhosa, Pastrana, and Cerqueira (2018). Films can also be used for controlling pathogenic microorganisms, improving food safety, or health promoting properties of the food (Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010; Sánchez-González, Saavedra, & Chiralt, 2013). Films made on the basis of edible biopolymers represent good opportunities for the development of new foods containing probiotics (Betoret, Betoret, Vidal, & Fito, 2011; Otoni, Espitia, Avena-Bustillos, & McHugh, 2016).

Kefiran is a GRAS (generally recognized as safe) status exopolysaccharide, present in kefir grains, composed by D-glucose and D-galactose residues (Abraham, Medrano, Piermaria, & Mozzi, 2010; Micheli, Uccelletti, Palleschi, & Crescenzi, 1999). Due to its structure and type of linkages cannot be hydrolyzed by gastrointestinal tract enzymes, which allow it to reach the small intestine where it exerts biological effects such as anti-cholesterolemic activity (Maeda, Zhu, Omura, Suzuki, & Kitamura, 2004), immunomodulatory activity (Medrano, Racedo, Rolny, Abraham, & Pérez, 2011), protection of the epithelium from the action of *Bacillus cereus* (Medrano, Pérez, & Abraham, 2008) and bifidogenic effect (Hamet, Medrano, Pérez, & Abraham, 2016). Kefiran is capable to form edible, transparent and homogeneous films, with good mechanical and barrier characteristics (Piermaria et al., 2011).

Whey proteins are widely used as food ingredients due to their nutritional and functional properties (Foegeding, Davis, Doucet, & McGuffey, 2002). They allow to obtain transparent films, with good mechanical and oxygen barrier properties (Ramos et al., 2012).

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Interaction between proteins and polysaccharides could influence film properties (Silva, Mauro, Gonçalves, & Rocha, 2016). Thereby combinations of these macromolecules can be used to generate mixed systems and thus optimize the characteristics of edible films (Prommakool, Sajjaanantakul, Janjarasskul, & Krochta, 2011) either keeping or adding their individual properties or due to the potential synergy between them (Vieira, da Silva, dos Santos, & Beppu, 2011).

Kluyveromyces marxianus CIDCA 8154 and *Lactobacillus paracasei* CIDCA 8339 were selected to be included in the films of present study by their probiotic characteristics. *L. paracasei* CIDCA 8339 has immunomodulatory effect, adhesion to intestinal epithelial cells *in vitro* capability (Bengoa et al., 2018 a & b) and it is able to diminish *Salmonella* invasion to the enterocytes *in vitro* (Zavala et al., 2016). *Kluyveromyces marxianus* CIDCA 8154 has capacity to adhere to epithelial intestine cells *in vitro* (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014), down-regulate intestinal epithelial innate response (Romanin et al., 2010) and whey fermented with this microorganism exhibited immunomodulatory capacity in response to a pro-inflammatory stimulus on Caco-2 CCL20:luc cells (Londero, Iraporda, Garrote, & Abraham, 2015).

The design of films as probiotic-release systems is a challenge since it must guarantee adequate viability in the final product and direct the probiotic specifically to the desired location. The objective of this work was to develop edible films from kefiran-whey proteins mixed system containing probiotic microorganisms. Besides, evaluate their characteristics and analyze the release of the microorganisms during the passage through the simulated gastrointestinal tract from recently made or stored films.

2. Materials and methods

2.1. Macromolecules

Kefiran was obtained as described in Piermaria, Diosma, Aquino, Garrote, and Abraham (2015) from kefir grains from CIDCA collection (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, Argentina). The polysaccharide concentration was normalized to 3.5% w/w and the solution was stored at −20 °C until its use.

Whey proteins isolate (wpi), Lacprodan DI-9224, containing 92% protein was kindly provided by *Arla Foods Ingredients* (Denmark).

2.2. Microorganisms

Lactobacillus paracasei CIDCA 8339 and *Kluyveromyces marxianus* CIDCA 8154 from CIDCA collection were used. The strains were maintained at −80 °C in 120 g L^{-1} non-fat milk solids. Lactobacilli were grown in De Man-Rogosa-Sharpe (MRS) agar (Difco, DIFCO, Detroit,USA) and yeast in Yeast Glucose Chloramphenicol (YGC) agar (Biokar Diagnostics, Beauvais, France), both under aerobic conditions at 30 °C for 48 h.

2.3. Macromolecules characterization

Kefiran molecular mass and concentration were achieved by HPLC according to Piermaria, Bengoechea, Abraham, and Guerrero (2016). For calibration, dextrans with molecular mass from 97.000 to 3.800.000 ALO-2770 (Phenomenex, Torrance, CA) were used as standards.

A thermal characterization of the whey proteins isolated was carried out on a Q100 differential scanning calorimeter (TA Instruments-Waters LLC, New Castle, USA). Around 15 mg of 20% w/w wpi in water suspension were weighed into aluminium pans and hermetically sealed. An empty pan was used as a reference. The scan was carried out from 0 to 100 °C at 10 °C/min. The denaturation temperature (Td), denaturation temperature range (ΔT_d), and denaturation enthalpy (ΔH_d) based on the protein mass within the suspension were obtained from thermograms using TA Universal Analysis 2000 software.

2.4. Preparation of film-forming dispersions

Solutions containing wpi, kefiran and glycerol (6%, 2% and 3.2% w/w respectively) in water including or not microorganisms were used as film-forming dispersions. For preparations, whey proteins were dissolved in water, combined with kefiran solution and then glycerol (Baker, Mexico) was added as plasticizer. The pH was adjusted to 7 using NaOH 5N and finally water was added to reach the desired concentration. The prepared solution was treated at 90 °C during 30 min to denature whey proteins.

Microorganisms inclusion was done growing *L. paracasei* CIDCA 8339 and *K. marxianus* CIDCA 8154 on agar plates, then collecting and suspending them individually in sterile phosphate buffer saline (PBS) up to optical density (OD) of 1 at 625 nm. Pellets harvested by centrifugation 5 min at 5000 *g* from each milliliter of each cell suspension (lactobacilli and yeast) were re-suspended in 5 g of film forming solution thermally treated before. The pH of film-forming dispersions was measured using a digital pHmeter (Hanna Instrument, USA), before and after microorganisms addition.

2.5. Film obtaining

Film-forming dispersions were placed into 50 mm diameter Petri dishes (0.18 g/cm^2) and dried during 16 h at 37 °C. The obtained films were removed from the plates and equilibrated at 75% of relative humidity (RH) and 20 °C prior to analysis.

2.6. Film characterization

Film thickness was evaluated using a digital coating thickness gauge (CM-8822 Digital meter Microprocessor) at 10 random positions, and average values were used in calculations.

Moisture content was determined by measuring weight loss of film samples undergone at 105 °C ± 1 °C until constant weight. Film samples were weighed with an accuracy of 0.00001 g, before and after drying. Results were expressed in grams of water per 100 g of sample.

Film color evaluation was carried out using the CIE Lab scale with a CR 300 Series Minolta colorimeter (Osaka, Japan). Film samples were placed over the white standard and evaluated in tenfold. The difference with standard value (ΔL*, Δa* and Δb*), was calculated for each parameter. The color difference (ΔE) was calculated according the equation:

$$\Delta E = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2}$$

Tensile properties were determined using a TA.XT2i–Stable Micro Systems texture analyzer (Surrey, England) with a tension grip system A/TG, according to the ASTM D882-91 method (1996). Tensile strength, elongation-at-break and elastic modulus were determined from the stress-strain curve.

Films microstructure was evaluated by scanning electron microscopy (SEM) and by environmental scanning electron microscopy (ESEM) using a 505 scanning electron microscope (Philips, Eindhoven, The Netherlands) and a Quanta 200 (FEI, Netherland) respectively. Small pieces of films were fixed on the support using double side adhesive tape. For SEM, samples were coated with a layer of 40 nm of gold and for ESEM no specific film preparation was necessary.

2.7. Gastrointestinal simulation passage of films

The survival of *L. paracasei* CIDCA 8339 and *K. marxianus* CIDCA 8154 included into the films was study under simulated gastrointestinal conditions. An *in vitro* model of gastrointestinal tract based on Grimoud et al. (2010) was employed. Free cells from fresh cultures of both

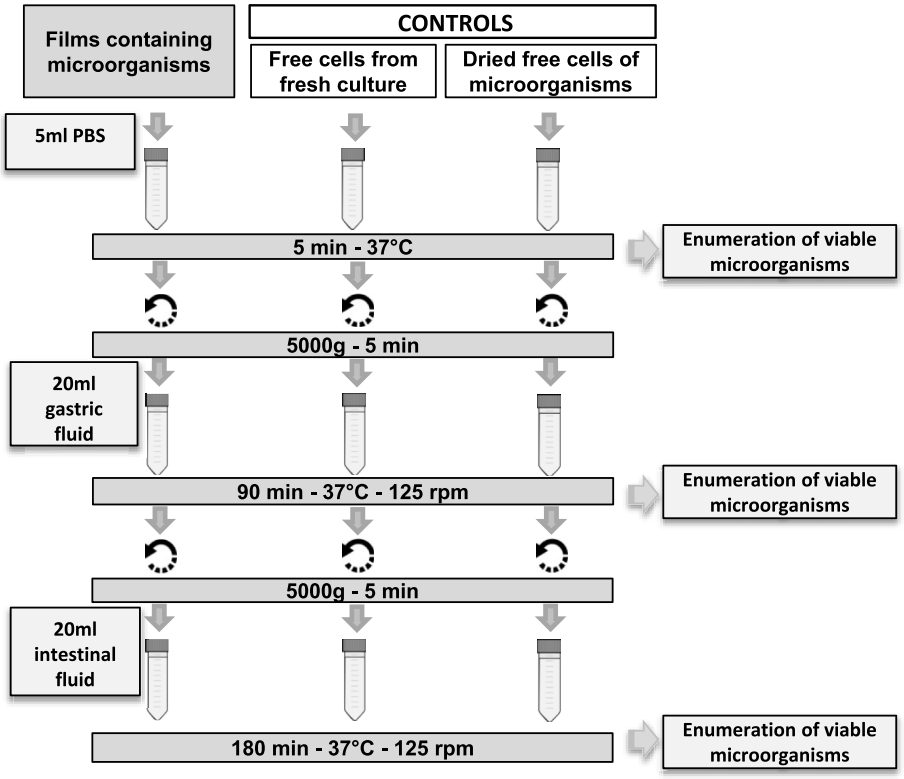

Fig. 1. Simulated gastrointestinal tract procedure used to evaluate viable microorganisms released from films or control suspensions.

microorganisms or suspensions of them previously dried in the same conditions as those used to obtain the films were included as controls (Fig. 1). These suspensions were carried out as the same way that film-forming dispersions including the same count of viable microorganisms, but using water.

Films or controls were hydrated in PBS and then exposed to simulated gastric fluid during 90 min followed by simulated intestinal fluid throughout 180 min. Prior to the addition of gastric or intestinal suspensions were centrifuged at 5000 *g* for 5 min. A ratio of 0.25, 1 and 1 ml of PBS, gastric or intestinal medium respectively per square centimeter of films, or the same number of microorganisms as those included in that portion of films, was employed. The simulation was carried out in an orbital shaker at 37 °C and 125 rpm (Fig. 1).

Simulated gastric fluid: NaCl 125 mM, KCl 7 mM, $NaHCO_3$ 45 mM, porcine pepsin 3 $g.L^{-1}$ (P7125 Sigma-Aldrich, Inc., St. Louis, MO, USA), pH 2.5.

Simulated intestinal fluid: NaCl 22 mM, KCl 3.2 mM, $NaHCO_3$ 7.6 mM, porcine pancreatin 1 $g.L^{-1}$ (P1625 Sigma-Aldrich, Inc., St. Louis, MO, USA), bile salts 1.5 $g.L^{-1}$ (48305 Sigma-Aldrich, Inc., St. Louis, MO, USA), pH 8.

The same procedure was repeated with films containing microorganisms as function of storage time during 60 days at 4 and 20 °C and 75% of relative humidity.

Enumeration of viable microorganisms before and after each gastrointestinal model step, was carried out by plating serial dilutions on the corresponding media and expressed as CFU per cm^2.

2.8. Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

Samples were analyzed by Tricine-SDS-PAGE using a 16%/6 M urea resolving gel, 10% spacer gel and a 4.0% stacking gel (acrylamide: N,N′-methylenebisacrylamide), on a vertical system OmniPAGE mini (Cleaver Scientific Ltd., Warwickshire, UK) according to the Schägger method (Schägger, 2006). Low-molecular-weight standards (GE-healthcare) containing phosphosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and a-lactalbumin (14.4 kDa) were used. Samples were dissolved in sample buffer (50 mM Tris-HCl, pH 6.8, 12% v/v glycerol, 4% w/v SDS and 0.01% w/v Coomasie blue G). For reducing conditions, 5% v/v 2-mercaptoethanol was added and samples were heated (100 °C, 3 min). Electrophoresis was done at 30 mA constant current for approximately 6 h. Gels were fixed and stained with Coomassie Brilliant Blue R.

2.9. Statistical analysis

The presented data were averaged from at least two independent experiments. Results were expressed as mean ± standard deviation. Differences were statistically tested by Analysis of Variance (ANOVA) and Fisher's least significant difference (LSD) mean discrimination test, using $\alpha = 0.05$ as level of significance using Statgraphics plus-software version 5.1 (StatPoint Technologies, Inc).

3. Results and discussion

3.1. Film-forming macromolecules and films characterization

Kefiran, wpi and glycerol as plasticizer were combined to generate the film-forming dispersions. Kefiran molecular weight was evaluated by gel permeation HPLC. A single peak with retention time corresponding to a molecular weight of $2.1.10^3$ kDa was detected, coinciding with previous references for kefiran (Piermaria et al., 2016). DSC analysis of wpi allowed the determination of a thermal transition at 75.6 ± 0.8 °C with an enthalpy of 3.0 ± 0.3 J/g corresponding to the denaturation of these proteins in concordance to previous published results (Fitzsimons, Mulvihill, & Morris, 2007). Taking into account these results, a heating step of 30 min at 90 °C during film obtaining process was included to complete denaturation of wpi.

Transparent and homogeneous films were obtained after drying

Table 1
Thickness, moisture, color and mechanical parameters of wpi-kefiran films containing or not *L. paracasei* CIDCA 8339 and *K. marxianus* CIDCA 8154.

Film type		Without microorganisms	Including microorganisms
Thickness (μm)		133 ± 19 [a]	143 ± 24 [a]
Moisture (%)		17,4 ± 2,2 [a]	16,5 ± 0,5 [a]
Color	ΔL	−1,5 ± 1,7 [a]	−2,0 ± 0,8 [a]
Parameters*	Δa	−0,6 ± 0,2 [a]	−0,62 ± 0,04 [a]
	Δb	2,3 ± 0,8 [a]	5,7 ± 0,6 [b]
	ΔE	3 ± 1 [a]	6,1 ± 0,8 [b]
Mechanical characterization	Tensile strength (MPa)	1,8 ± 0,2 [a]	1,8 ± 0,3 [a]
	Elastic modulus (MPa)	9,1 ± 2,2 [a]	8,5 ± 1,8 [a]
	Elongation at break (%)	80,5 ± 19,3 [a]	105,2 ± 30,7 [a]

*ΔL, Δa, Δb indicate CIE Lab values differences of lightness, redness and yellowness respectively, over the white background. ΔE indicates color difference. Different letters indicate significant differences for each parameter between films with and without microorganisms ($p \leq 0.05$).

film-forming dispersions. The inclusion of microorganisms did not alter its pH (7.0 ± 0.1). Films containing or not microorganisms were easily removed from the Petri dishes and they presented homogeneous appearance. Results of thickness, moisture, color and mechanical characterization of obtained films are shown in Table 1.

The thickness of films with or without microorganisms was 143 ± 24 and 133 ± 19 μm respectively and there were no differences between them (Table 1). Pereira et al. (2016) also found similar thickness for wpi films including or not *Bifidobacterium animalis* or *Lactobacillus casei*. Likewise, moisture values of films were also not modified by the inclusion of microorganisms. Values were in the same range than those found for other edible macromolecules mixed films including probiotics like sodium alginate-whey proteins films (Soukoulis et al., 2014) or the corresponding to the pullulan-starch films studied by Kanmani and Lim (2013).

In the color evaluation (Table 1), no significant differences were found in luminosity (△L) or in the color parameter a (△a) between the films with and without microorganisms. A difference in the color parameter b (△b) was found and it correlates with a slightly yellow appearance in the films containing microorganisms, generating a change in the total color difference (△E). Comparing with Pereira et al. (2016) who studied films based on wpi including or not microorganisms, wpi-kefiran films presented lower values of △E.

As regards mechanical characterization showed in Table 1, the wpi-kefiran films presented intermediate values of the three mechanical parameters between those corresponding to kefiran films (Piermaria et al., 2011) and those corresponding to films constituted only by wpi (Pereira et al., 2016).

When microorganisms were included in the matrix no changes in mechanical parameters of wpi-kefiran films were found. A similar trend was observed for the pea protein or sodium caseinate edible films after the inclusion of microorganisms (Gialamas et al., 2010; Sánchez-González et al., 2013).

In the microstructural analysis carried out by both SEM and ESEM, all films showed homogeneous appearance and roughness surface and there was coherence between images obtained by both microscopy techniques (Fig. 2). In the cross section of films containing microorganisms, footprints compatible in shape and size with yeasts could be observed (Fig. 2a). In the ESEM image the yeasts presence becomes evident (Fig. 2c, right side). Microorganisms were entrapped in the matrix film without changing its normal conformation in concordance to Tavera-Quiroz et al. (2015). Regarding films constituted only by kefiran (Piermaria et al., 2015), the incorporation of wpi proteins to the formulation increases surface roughness of films. The roughness of wpi-kefiran films might be due to interactions between proteins and polysaccharide.

Ability of wpi-kefiran films as carrier of probiotics through the gastrointestinal tract was evaluated by performing a simulation model. The behavior of films was analyzed focusing in two topics: the effect of gastrointestinal model on proteins of the matrix and the release of viable microorganisms from the films.

Degradation of proteins in the film matrix during gastrointestinal simulation was analyzed by Tricine-SDS-PAGE electrophoresis. Fig. 3 shows the protein pattern of the film before and after gastrointestinal treatment. In lane 1 bands corresponding to the low molecular weight pattern are shown. Profile observed in lane 2 corresponds to film hydrated in PBS and it is due to constituent matrix proteins, where are evident bands of β-lactoglobulin (18 kDa), α-lactalbumin (14 kDa) and serum albumin (66 kDa). After 45 and 90 min of gastric treatment (lanes 3 and 4) a slight decrease in bands intensity corresponding to the major proteins present in the wpi is observed, indicating that only a low degree of proteolysis occurred. Lanes 5, 6 and 7 show the results of the application of intestinal simulation during 60, 120 and 180 min respectively. In these lanes, a gradual decrease in the intensity of the bands corresponding to the proteins α-lactalbumin and β-lactoglobulin with the concomitant appearance of lower molecular weights bands was observed. According to these results, although there is an onset of degradation of the matrix during the gastric phase, the main degradation occurs in the intestinal phase.

L. paracasei CIDCA 8339 and *K. marxianus* CIDCA 8154 released from films after each stage of gastrointestinal model were analyzed (Figs. 4 and 5). Suspensions of both microorganisms obtained from fresh cultures or subjected to the drying conditions used to obtain the films were employed as controls. Films or controls were submitted to a first stage of hydration in PBS, followed by a gastric simulation step and finally a stage of intestinal simulation (Fig. 1). After each step, viable lactobacilli and yeasts were enumerated and compared with initially number included in the film-forming dispersion.

Results concerning to the behavior of viable *K. marxianus* CIDCA 8154 released after each stage of gastrointestinal model are shown in Fig. 4. The initial number of microorganisms included in film formulation is presented as a dotted line in the figure. When microorganisms suspension from fresh cultures were submitted to the gastrointestinal sequence, no loss of yeast viability was observed due to the pH or to the action of gastric and intestinal enzymes (Fig. 4a), indicating that *K. marxianus* CIDCA 8154 is not affected by gastrointestinal conditions. The control suspension containing free yeast previously subjected to the drying process evidenced a significant decrease in the number of viable microorganisms regarding the formulation (Fig. 4b). The drying stage affected yeast viability but the subsequent gastric and intestinal simulation treatments did not lead to an additional decrease in the number of viable *K. marxianus*. Yeasts that resist drying treatment would resist gastrointestinal passage without loss of viability. When films including microorganisms were submitted to gastrointestinal conditions a different behavior regarding controls of *K. marxianus* CIDCA 8154 was observed (Fig. 4c). After hydration of films in PBS a low number of viable microorganisms were released. Nevertheless, after subsequent gastric and intestinal stages the number of viable yeasts released ($7.1.10^5$ CFU/cm^2) was not different to that originally included in the formulation indicating that it is necessary to

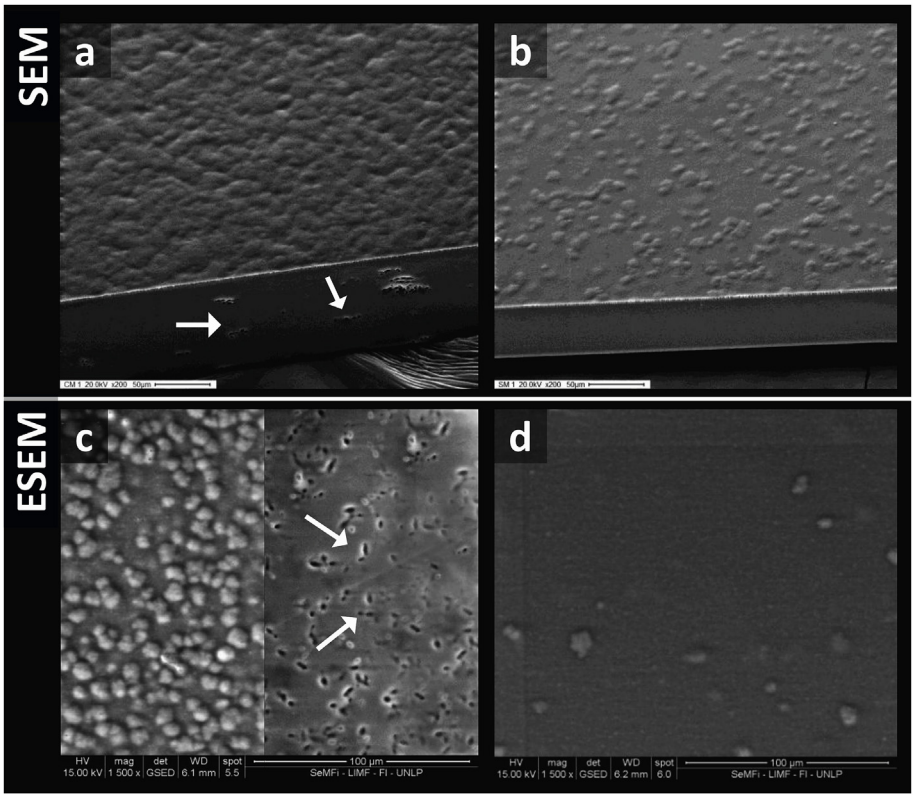

Fig. 2. Micrographs obtained by SEM (upper line) and ESEM (bottom line) of films containing (a, c) or not (b, d) *L. paracasei* and *K. marxianus*. Image c is a merge of two images: the left corresponds to the film side exposed to the air and at the right is shown the side in contact with Petri dish, during drying step. Arrows show footprints compatible in shape and size with yeasts included in the films.

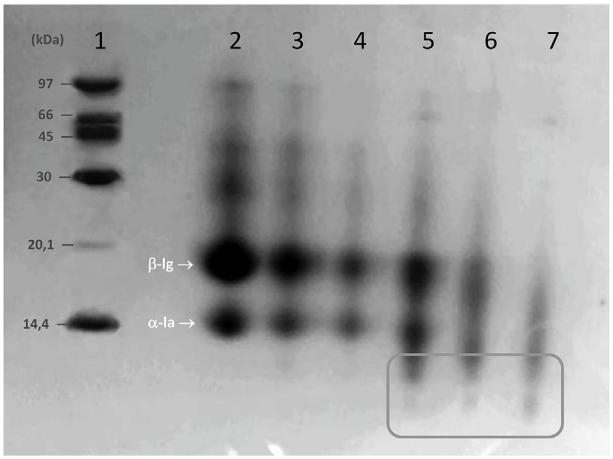

Fig. 3. Tricine-SDS-PAGE electrophoresis gel of proteins in films before and through gastrointestinal simulation. Lane 1: low molecular weight pattern. Lane 2: before gastrointestinal treatment. Lanes 3 and 4: 45 and 90 min of gastric treatment. Lanes 5, 6 and 7: 60, 120 and 180 min of intestinal treatment, respectively. White arrows indicate bands correspondent to β-lactoglobulin and α-lactalbumin, major proteins present in whey protein isolate. Grey rectangle signalizes hydrolyzed products from film protein digestion appearing in lines 5, 6 and 7.

partially hydrolyze wpi to release yeast included into the matrix. Gastrointestinal enzymes progressively degrade matrix being proteolysis accentuated with the increase of time of exposure to the intestinal environment, allowing the yeast release to the medium. According to those results, film matrix would protect *K. marxianus* CIDCA 8154 from stress damage during drying step directing them viable to the intestine.

Drying treatment was responsible of yeast viability loss. However, when the yeasts are immersed in the wpi-kefiran matrix they resist drying process. This observation, that is in concordance with Soukoulis et al. 2014, would indicate that high recovery of viable cells included in the film matrix after being submitted to gastrointestinal conditions could be due to these microorganisms interacting with wpi-kefiran matrix, which would protect them against damages due to oxidative and osmotic stress they are subjected during drying and film obtaining process.

The results corresponding to the viability maintenance of *L. paracasei* CIDCA 8339 during gastrointestinal simulation are presented in Fig. 5. When control suspension containing microorganisms from fresh culture was submitted to the simulated gastrointestinal passage (Fig. 5a), it was observed that hydration in PBS had no effect on the viability. After gastric simulation stage, the number of viable bacteria decreased more than five logarithmic cycles, indicating that the studied *L. paracasei* is very sensitive to this condition, and after intestinal step no viable bacteria was obtained.

When gastrointestinal simulation was carried out on the control constituted by dried bacteria (Fig. 5b), count of viable bacteria after hydration was lower than the initial number represented by the dotted line. Subsequently, successive simulation stages led to an additional decrease in observed viability.

Fig. 5c shows the number of viable *L. paracasei* CIDCA 8339 recovered from films after each step of gastrointestinal simulation. When films were hydrated in PBS the number of viable bacteria recovered was significantly lower than the included in formulation. After gastric step simulation, this number was increased and when intestinal stage was completed the count of viable bacteria was in the same order than the initially incorporated. Therefore, this results indicates that during hydration in PBS microorganism are maintained associated, at least in part, to the films and those interactions between bacteria cells and surrounding film matrix, could result in increasing cell-protective effects during drying step and against damages produced by gastrointestinal tract conditions. Previous results have demonstrated that anionic polysaccharide did not protect *Lactobacillus rhamnosus* GG from drying stress whereas the inclusion of whey protein concentrate in formulation improves their survival (Soukoulis, Behboudi-Jobbehdar, Macnaughtan, Parmenter, & Fisk, 2017). The chemistry of the film is a

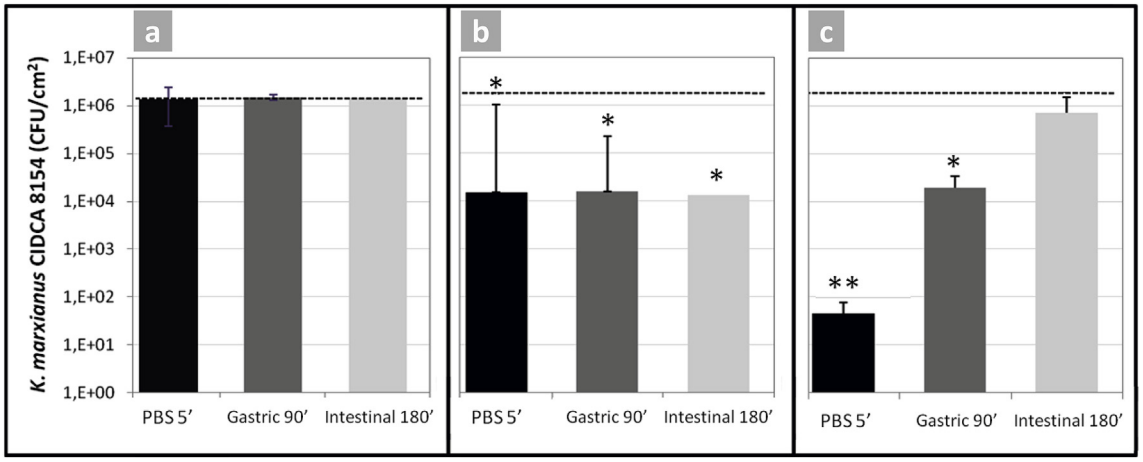

Fig. 4. Viable *K. marxianus* CIDCA 8154 released after each gastrointestinal stage from: fresh culture control (a), dried suspension control (b) or wpi-kefiran film (c) containing lactobacilli and yeasts. Dotted lines show the concentration of yeast in the initial suspensions. Asterisks indicate significant differences with respect to that concentration ($p \leq 0.05$). The error bars represent standard deviation.

critical factor for bacterial survival during processing and ingestion, in this sense wpi-kefiran film not only protect microorganisms against processing stress but also to acid and bile stress occurring during probiotic ingestion.

Films containing microorganisms were stored at 4 and 20 °C and gastrointestinal simulation was performed at different times during the storage. The number of viable microorganisms recovered from films after sequential stages application is shown in Fig. 6. The count of *K. marxianus* recovered viable after gastrointestinal model was maintained during the first 9 days regardless of the storage temperature. Then, concentration decreased progressively until around 2 logarithmic cycles when films were kept at 4 °C and more than 5 logarithmic cycles in the storage at 20 °C after 57 days (Fig. 6a).

The evaluation of *L. paracasei* indicated that when films were stored at 20 °C the count of recovered bacteria after gastrointestinal simulation remained like the corresponding to recently obtained films during the first 9 days. After 23 days, the viable count was significantly reduced and at day 57 there was only $4.4.10^2$ CFU/cm^2. Distinctively, when films were stored at 4 °C was possible to recover the same bacteria count after 57 days than the corresponding to recently obtained films (Fig. 6b). These results are in concordance to previous works about storage of microorganisms included in edible films, in which have been reported that the higher viability observed at 4 °C, compared to that observed at 25 °C, is a consequence of low levels of bacterial metabolism when they are maintained at low temperatures (Gialamas et al., 2010).

The developed edible wpi-kefiran films satisfy the requires for new materials used to include probiotics since they were capable to protect the selected microorganisms during elaboration process and to maintain their viability through the gastrointestinal passage in order to reach the intestine where they perform their biological effect. In addition, the polysaccharide kefiran has bifidogenic and immunomodulatory capacity that would contribute to the benefit for the health consumers attributed to the probiotics.

4. Conclusions

Wpi-kefiran films were investigated as new alternative for the delivery of probiotics. The developed edible wpi-kefiran films presented good optical and mechanical properties to be used in functional foods and those characteristics were maintained when microorganisms were included.

The films resulted to be a good strategy since they were able to protect *K. marxianus* CIDCA 8154 from stress damages due to drying process and *L. paracasei* CIDCA 8339, against processing as much as gastrointestinal passage injuries.

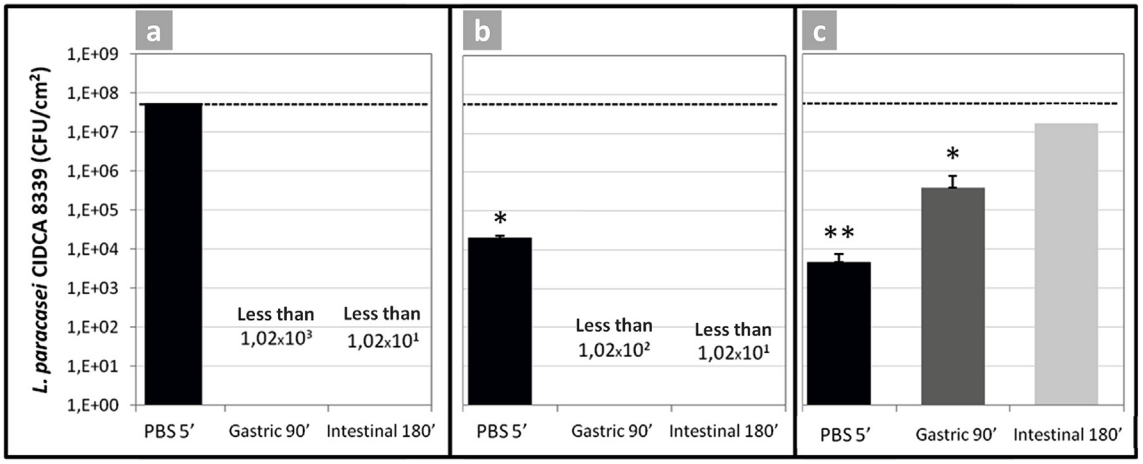

Fig. 5. Viable *L. paracasei* CIDCA 8339 released after each gastrointestinal stage from: fresh culture control (a), dried suspension control (b) or wpi-kefiran film (c) containing lactobacilli and yeasts. Dotted lines show the concentration of lactobacilli in the initial suspensions. Asterisks indicate significant differences with respect to that concentration ($p \leq 0.05$). The error bars represent standard deviation.

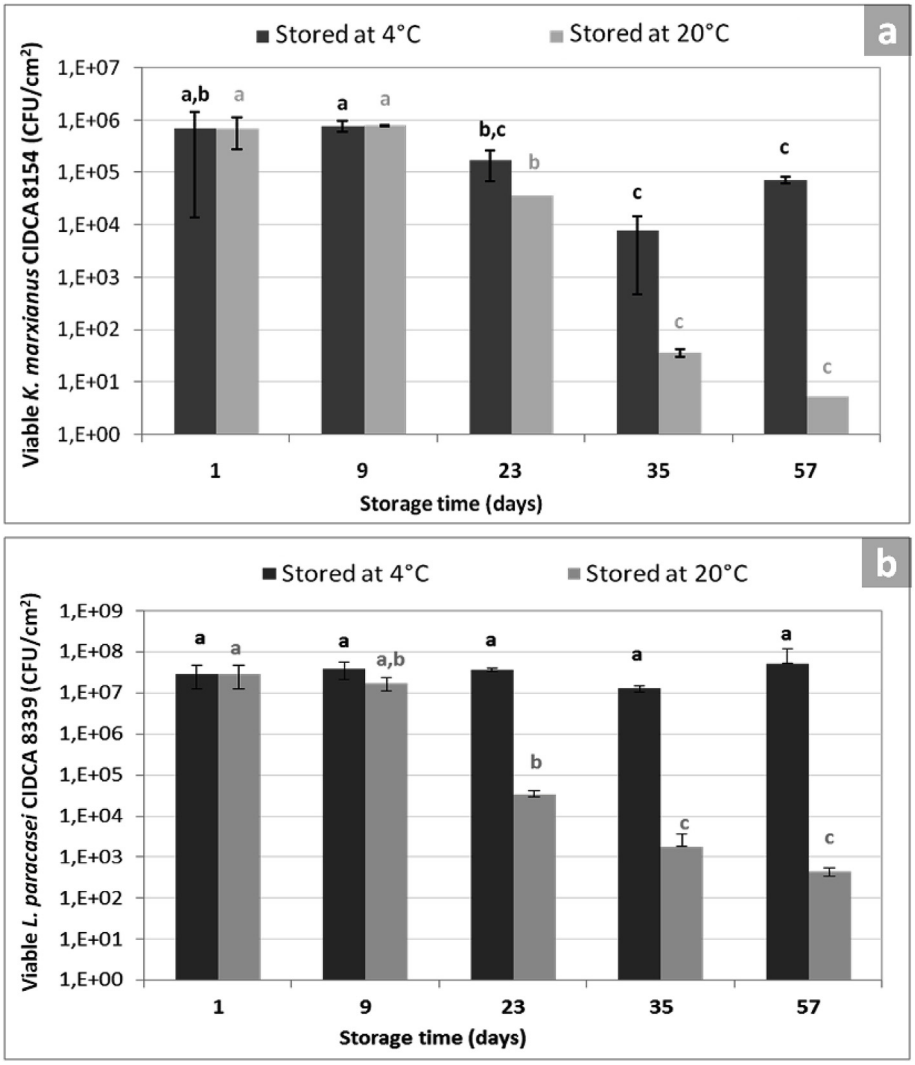

Fig. 6. Viable *K. marxianus* 8154 (a) or *L. paracasei* CIDCA 8339 (b) released from films submitted to gastrointestinal model at different times during storage at 4 °C (dark bars) or 20 °C (light bars). Different letters indicate significant difference within same storage condition ($p \leq 0.05$). The error bars represent standard deviation.

Targeted delivery of probiotics was possible because of the matrix degradation take place at intestinal conditions, since release of microorganisms from the film is not possible during gastric stage.

Temperature of 4 °C resulted to be an appropriate condition to store these films allowing, at least during 57 days, an excellent maintenance of both microorganisms viability after the passage through gastrointestinal tract.

The present study suggests the potential of wpi-kefiran films as alternative for probiotics delivery in the development of functional products, improving their viability through industrial processing, storage and consumption.

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