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Edible kefiran films as vehicle for probiotic microorganisms

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ABSTRACT

In the presented work, we have developed edible kefiran films containing *Lactobacillus plantarum* CIDCA 8327 and *Kluyveromyces marxianus* CIDCA 8154. Thickness, moisture content and optical properties of films were not altered by the incorporation of microorganisms. During the film obtaining, lactobacillus concentration decreased one logarithmic cycle and no loss on yeast viability was detected. Both microorganisms included in glycerol plasticized kefiran films successfully maintained their viability during storage. After 35 days at 20°°C *L. plantarum* viability decreased less than 1.3 logarithmic cycles and *K. marxianus* 0.7 logarithmic cycles.
The high susceptibility of free cells of lactobacilli to acid was slightly diminished by the inclusion in the kefiran matrix. Inclusion of *K. marxianus* CIDCA 8154 significantly improved their survival to the sequential acid–bile treatment (36.3%) compared with the corresponding free cells (8.7%). The obtained edible kefiran films have great potential as an alternative way for probiotic delivery.
Industrial relevance: The edible kefiran films containing probiotics presented in this manuscript are interesting matrices for probiotic administration since the neutral polysaccharide kefiran, produced by lactic acid bacteria, has health-promoting properties itself. Films can be obtained simply and inexpensively. They are transparent, thin and have good mechanical properties among those constituted by biomacromolecules. Likewise, kefiran films can preserve the viability of probiotics in non-refrigerated conditions.

1. Introduction

The development of innovative functional products to satisfy the demands related with food quality and health-promoting characteristics has gained the interest of industries and researchers (Betoret, Betoret, Vidal, & Fito, 2011). Several health-related effects were associated with the intake of foods containing probiotics. The term probiotics was technically defined by an expert committee as "live microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (FAO/WHO, 2002).

Although fermented dairy products remain the most important food for delivering probiotic microorganisms, the exploration of alternative matrices is a constant challenge for researchers and industries (Rivera-Espinoza & Gallardo-Navarro, 2010). Edible films are an interesting alternative because they may contain a large amount of cells in a very low weight and do not necessarily require refrigeration (Martín-Belloso, Rojas-Graü, & Soliva-Fortuny, 2009; Quezada-Gallo, 2009).

Food grade polymer with health promoting properties could represent an advantageous alternative for the development of functional foods. Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter and Fisk (2014) proposed the use of prebiotic edible films as effective vehicles for encapsulating probiotic living cells. In this regard, kefiran, the neutral polysaccharide produced by lactic acid bacteria (LAB) included into kefir grains is a promising polysaccharide that has functional and technological properties. Kefiran is capable of forming transparent, flexible, homogeneous and extremely thin edible films which show adequate barrier and mechanical properties (Piermaria, Pinotti, García, & Abraham, 2009). In addition, several biological effects are associated with kefiran consumption such as the antagonic activity against virulence factors of *Bacillus cereus* (Medrano, Pérez, & Abraham, 2008) and immunomodulatory activity in vitro and in vivo (Abraham, Medrano, Piermaria, & Mozzi, 2010; Furuno & Nakanishi, 2012; Medrano, Racedo, Rolny, Abraham, & Perez, 2011) and promote in vitro growth of a *Bifidobacterium bifidum* strain (Serafini et al., 2014). Because of both the health-promoting properties and the capability of the polysaccharide to form edible films, kefiran becomes a promising matrix for developing functional foods.

The present work attempts to obtain kefiran films including potentially probiotic lactic acid bacteria and yeasts. For that purpose, two strains isolated from kefir grains were selected: *Lactobacillus plantarum* CIDCA 8327 for its high adhesion ability to epithelial Caco-2

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cells and its ability to inhibit the growth of *Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Gallinarum, *S. enterica* serovar Enteritidis, *Escherichia coli* and *Shigella sonnei* (Golowczyc et al., 2008); *Kluyveromyces marxianus* CIDCA 8154 because of its capacity to adhere to epithelial intestine-derived cells in vitro (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014) and down-regulate intestinal epithelial innate response (Romanin et al., 2010) and whey fermented with these microorganisms exhibited immunomodulatory capacity in response to a pro-inflammatory stimulus in vitro on Caco-2 CCL20: luc cells (Londero, Iraporda, Garrote, & Abraham, 2015). In addition, the characterization of the resulting films was also performed.

A probiotic has to reach the small intestine viable overpassing detrimental effects that take place due to food processing and storage as well as due to the passage through the gastrointestinal tract (Fu & Chen, 2011). Investigation of the acid tolerance and bile resistance of potentially probiotic microorganisms included in kefiran edible films and the amount of viable *L. plantarum* CIDCA 8327 and *K. marxianus* CIDCA 8154 remaining during storage were also included as objectives of the present study.

2. Materials and methods

2.1. Isolation of kefiran

The polysaccharide kefiran was obtained from kefir grains of the CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, Argentina) collection. A weighed amount of kefir grains CIDCA AGK1, obtained by successive subcultures in commercial ultra-high temperature (UHT) low fat milk obtained from Sancor (Santa Fe, Argentina), was treated in boiling water (1:10 *w*/*w*) for 30 min with discontinuous stirring and then was sterilized at 121°°C for 15 min. The product was centrifuged at 10,000 *g* for 20 min at 20°°C (Avanti J25 Beckman Coulter Inc. centrifuge, Palo Alto, California). The resulting supernatant was transferred to sterile flasks and sugar and protein concentration were measured by the anthrone (Southgate, 1976) and Bradford (Bradford, 1976) methods respectively. Kefiran concentration was adjusted to 2% *w*/*w* (protein concentration lower than 0.2% *w*/*w*) and the solution stored at −20°°C.

2.2. Microorganisms

L. plantarum CIDCA 8327 and *K. marxianus* CIDCA 8154 from the 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 (Biokar Diagnostics, Beauvais, France) and yeast in yeast glucose chloramphenicol (YGC) agar (Merck, D-64271 Darmstadt, Germany), both under aerobic conditions at 30°°C for 48 h.

2.3. Preparation and characterization of film-forming dispersions

Different film-forming dispersions, based on inclusion or not of microorganisms and the plasticizer glycerol, were used: kefiran (K), kefiran containing lactobacilli (K-LAB), kefiran containing yeast (K-Yeast), kefiran containing a mix of lactobacilli and yeasts (K-Mix), kefiran with glycerol 10% *w*/*w* (10 g per 100 g of polysaccharide) (K_{gly}) and kefiran with glycerol 10% *w*/*w* containing a mix of lactobacilli and yeasts (K_{gly}-Mix).

For microorganism inclusion in film-forming dispersions *L. plantarum* CIDCA 8327 and *K. marxianus* CIDCA 8154 grown on agar plates, as described previously, were collected and suspended in sterile phosphate buffered saline (PBS) to an optical density (OD) at 625 nm of 1. One milliliter of each cell suspension were harvested by centrifugation during 5 min at 5000 *g*, washed and re-suspended in 10 mL of 2% *w*/*w* kefiran solution.

To determine the concentration of viable microorganisms dilutions 1:10 of film-forming dispersions in tryptone (1 g L^{-1}) were vortexed during 3 min with glass beads. Then, serial dilutions (1:10) in tryptone were performed and plated on MRS agar, for lactic acid bacteria, and on YGC agar, for yeasts. Plates were incubated for 48 h at 30°°C before colonies were counted.

The pH of the film-forming dispersions was measured using a digital pH meter (Hanna Instrument, USA), before and after microorganism addition.

Rheological characterization of the film-forming dispersions was performed in Haake ReoStress 600 (Thermo Haake, Karlsruhe, Germany) in rotational mode at controlled constant temperature 20°°C using a 1 mm gap plate–plate sensor system PP35. Shear stress (σ) was determined as a function of shear rate (γ) using the following program: 2 min to reach the maximum shear rate (500 s^{-1}), then it was maintained during 1 min and finally it was allowed to reach 0 shear rate in 2 min. Rheological behavior was correlated by the Ostwald–de Waele model:

$$\sigma = K_x \dot{\gamma}^n$$

where K is the consistency index (Pa s^n), γ is the shear rate (s^{-1}) and n is the flow index (dimensionless). The apparent viscosity (mPa s) values were calculated at 500 s^{-1}.

2.4. Film obtaining and characterization

Films were obtained by casting of 3.5 g of homogenized kefiran film-forming dispersions into Petri dishes (diameter 50 mm) and dried at 37°°C in a controlled temperature chamber for 11–14 h. The obtained films were carefully removed from the plates and equilibrated at 20°°C and 75% relative humidity (RH) for 5 h prior to analysis. The six different obtained films were named like corresponding film-forming dispersions (K, K-LAB, K-Yeast, K-Mix, K_{gly} and K_{gly}-Mix).

Film thickness was measured with a digital coating thickness gauge Check Line DCN-900 (New York, USA) with an accuracy of 0.1 μm at fifteen random positions on each film.

Moisture content of films was determined by measuring the weight loss, upon drying in an oven at 105°°C ± 1°°C until constant weight (dry sample weight). Film samples were weighted before and after drying using a digital balance (AE240 Mettler, Toledo, Spain) with an accuracy of 0.00001 g. Samples were analyzed at least in triplicate and results were expressed in grams of water per 100 g of sample.

Film color evaluation was performed using a CR 300 Series Minolta colorimeter (Osaka, Japan) calibrated with a white standard (Y = 93.2, x = 0.3133, y = 0.3192). Lightness (L*) and chromaticity parameters a*(red–green) and b* (yellow–blue) were measured using the CIE Lab scale. Film samples were placed over the white standard and evaluated in ten random positions. The difference with average standard value was calculated for each parameter. From these differences, ΔL*, Δa* and Δb*, the color difference (ΔE) was calculated using the following equation.

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

For scanning electron microscopy (SEM) studies, film pieces were immersed in liquid nitrogen and then samples were fractured and mounted on aluminum stubs using a double-sided tape. Later, they were gold coated with a layer of 40 nm to 50 nm of thickness in the sputter coater (Balzers, Bal Tec AG, Liechtenstein) and observed using a 505 scanning electron microscope (Philips, Eindhoven, The Netherlands) with an accelerating voltage of 20 kV to 25 kV.

The surface morphology of the film samples was analyzed by atomic force microscopy (AFM). Pieces of different samples were fit into AFM imaging, and double-sided tape was used to attach them onto

Table 1
Humidity, thickness, color and roughness parameters of films.

Film	Humidity (% *w*/*w*)	Thickness (μm)	Color				Sa (nm)	Sq (nm)
			ΔL*	Δa*	Δb*	ΔE		
K	10.2 ± 1.2^{a}	$15.1 \pm 3.9^{a,b}$	0.00 ± 0.09^{a}	0.00 ± 0.02^{a}	0.00 ± 0.11^{a}	0.12 ± 0.06^{a}	23.3 ± 3.7^{a}	28.8 ± 3.5^{a}
K-Yeast	12.1 ± 2.8^{a}	19.3 ± 6.9^{b}	-0.06 ± 0.46^{a}	-0.45 ± 0.06^{b}	1.74 ± 0.28^{b}	1.85 ± 0.30^{b}	$35{,}0 \pm 7.6^{b,c}$	$44{,}5 \pm 9.0^{b,c}$
K-LAB	$10.0 \pm 0.2^{a,b}$	13.2 ± 4.8^{a}	0.19 ± 0.53^{a}	-0.22 ± 0.02^{c}	0.69 ± 0.11^{c}	0.69 ± 0.11^{c}	$22{,}2 \pm 5.6^{a}$	$27{,}2 \pm 6.6^{a}$
K-MIX	7.7 ± 0.9^{b}	$19.1 \pm 3.6^{a,b}$	-0.21 ± 0.43^{a}	-0.43 ± 0.04^{b}	1.42 ± 0.11^{d}	$0.89 \pm 0.19^{b,d}$	$41{,}7 \pm 3.6^{c}$	$51{,}1 \pm 4.6^{c}$
K_{gly}	12.1 ± 0.6^{a}	$17.2 \pm 4.0^{a,b}$	-0.16 ± 0.17^{a}	-0.01 ± 0.02^{a}	0.11 ± 0.12^{a}	0.24 ± 0.15^{a}	31.1 ± 6.2^{b}	40.1 ± 8.3^{b}
K_{gly}-Mix	11.3 ± 0.9^{a}	$17.3 \pm 5.4^{a,b}$	-0.44 ± 0.44^{a}	-0.15 ± 0.07^{d}	1.13 ± 0.47^{d}	$1.26 \pm 0.55^{c,d}$	$34{,}9 \pm 1.3^{b,c}$	$43{,}6 \pm 2.2^{b,c}$

ΔL*, Δa* and Δb* are the differences of luminosity and color parameters between the standard white surface and the same surface cover by samples. ΔE is the color difference calculated according to the equation in the text. Sa and Sq are average roughness and root-mean-square roughness respectively. Values are given as mean ± standard deviation. Different letters within the columns correspond to significantly different values with $p \leq 0.05$.

the sample stage. All film samples were scanned in tapping mode commanded by a Nanoscope V control unit, from Veeco Instruments (Santa Barbara, CA, USA). The probe used from Bruker (Santa Barbara, CA, USA) model TESPA (cantilever resonance frequency: 330–360 kHz, force constant 20 N/m to 80 N/m; tip radius 8 nm to 12 nm). Typical scan rates were 1 Hz to 1.5 Hz. Data results were transformed into 3D images and roughness values of the films were calculated using Nanoscope 7.30 software. Two statistical parameters were calculated: average roughness (*Sa*: average of the absolute value of the height deviations from a mean surface), and root-mean-square roughness (*Sq*: root-mean-square average of height deviations taken from the mean data plane). The maximun difference of height (ΔH_{max}) was also evaluated in samples.

The tensile properties of the films were determined according to the ASTM D882-91 method (1996) using a tension grip system A/TG and probes of 6 cm length and 0.7 cm width. Tensile strength, elongation-at-break and modulus of elasticity were determined from the stress–strain curve using a TA.XT2i-Stable Micro Systems texture analyzer (Surrey, England). Each informed value corresponded to the average of at least six determinations.

To determine the number of viable microorganisms included into kefiran films, they were placed in 25 mL of PBS, homogenized in vortex with sterile glass beads (diameter 5 mm) for 3 min and then serially diluted in tryptone (1 g L^{-1}). The number of microorganisms was expressed as CFU for cm^2 of film.

The viability of microorganisms was studied during film storage at 20°°C. At 8, 20, 35 and 60 days, samples of each film were removed from the Petri dishes and analyzed according to the methodology described above.

2.5. Acid and bile resistance of microorganisms included in films

Acid, bile and sequential acid–bile tolerance of microorganisms included in films was assayed. Samples of films were placed in 5 mL of PBS during 5 min, then they were centrifuged at 5000 *g* and the pellets were submitted to 25 mL of each of the following solutions modified from Versantvoort, Oomen, Van de Kamp, Rompelberg, and Sips (2005) at 37°°C during 1 h with orbital agitation at 100 rpm.

•Acid solution: NaCl 2.75 g/L; NaH_2PO_4 0.27 g/L; KCl 0.82 g/L; $CaCl_2 \cdot 2H_2O$ 0.40 g/L; NH_4Cl 0.31 g/L, HCl 7.75 g/L. pH 2.09.

•Bile solution: NaCl 5.78 g/L; $NaHCO_3$ 3.77; KCl 0.62 g/L; HCl 0.18 g/L; $CaCl_2 \cdot 2H_2O$ 0.19 g/L; KH_2PO4 0.05 g/L; $MgCl_2$ 0.03 g/L, bilis extract 18 g/L. pH 8.2.

•PBS solution was used as control.

For the evaluation of the sequential acid–bile tolerance, film samples subject to acid solution were centrifuged 5 min at 5000 *g* and bile solution was added to the pellet and kept at 37°°C during one additional hour. After the exposure time glass beads were added to each sample and mixed in vortex during 3 min. The concentration of viable microorganisms and survival in each studied condition were determined as described previously by plating serial dilutions on the corresponding media. The number of viable cells was expressed as CFU for cm^2.

Acid, bile and sequential acid–bile tolerance was evaluated for free viable cells of each studied microorganism. Suspensions of lactobacilli

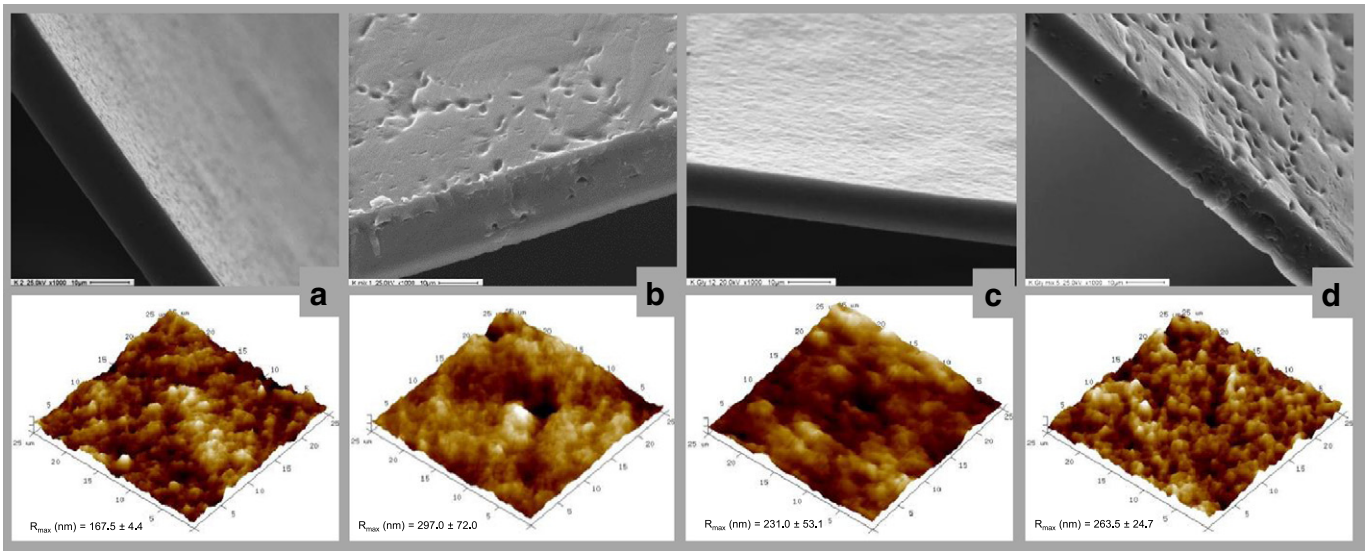

Fig. 1. Micrographs obtained by SEM or AFM of unplasticized kefiran films without microorganisms, K, (a) or containing lactic acid bacteria and yeast (K-Mix) and glycerol plasticized kefiran films without microorganisms, K_{gly} (c) or containing both lactic acid bacteria and yeast, K_{gly}-Mix (d). Magnification used was 1000×.

and yeast in PBS medium with the same number of viable microorganisms contained in the films were submitted to acid, bile or sequential acid–bile solutions as was described above.

2.6. Statistical analysis

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

3. Results and discussion

3.1. Film-forming dispersions and film characterization

As the rheology of film-forming dispersion affects the final characteristics of edible films (Peressini, Bravin, Lapasin, Rizzotti, & Sensidoni, 2003), the flow curves of film-forming dispersions were analyzed and the apparent viscosity, at 500 s^{-1}, was calculated for each dispersion. The Ostwald–de Waele model fitted satisfactorily ($r^2 > 0.99$) the experimental plots of shear stress (σ) versus shear rate (γ) in the evaluated shear rate range. The consistency index values were 0.0114 Pa s^n to 0.0118 Pa s^n. All film-forming dispersions showed a pseudoplastic behavior according to the flow index values (n) that varies between 0.9443 and 0.9608. The apparent viscosities of film-forming dispersions varied from 8.17 mPa s to 8.47 mPa s and the presence of glycerol or microorganisms did not modify significantly the rheological parameters.

The pH of the film-forming dispersions before addition of microorganisms was 4.1 ± 0.1. Contrary to reports by Kanmani and Lim (2013), the microorganism inclusion did not alter the pH.

Kefiran films with or without microorganisms were easily removed from the Petri dishes and they presented smooth surfaces and a transparent and homogeneous aspect. The thickness of films varied between 13.2 μm and 19.3 μm, the moisture content was between 7.7% *w/w* and 12.1% *w/w* and no significant changes in these characteristics were observed when microorganisms were included in the films (Table 1).

Film evaluation revealed that the color differences were all close to zero (Table 1). Differences in thickness could affect color parameters, mainly the luminosity values. Taking into account that thickness of films containing yeast, lactobacilli or both did not differ significantly from the value of the corresponding kefiran film without microorganisms, obtained color parameters are comparable. For films containing microorganisms lower Δa^* and higher Δb^* values were found indicating a negligible tendency to green and yellow respectively. When the color difference, ΔE, was calculated, the higher values were obtained for K-Yeast, K-Mix and K_{gly}-Mix films indicating that yeasts were the main responsible of this feature. Since the human eye can appreciate differences in color "as obvious" when the parameter ΔE is higher than 3 (Bodart, de Peñaranda, Deneyer, & Flamant, 2008), the inclusion of yeasts led to color changes that would be appreciated as minor color differences by consumers ($\Delta E \leq 1.85$).

Microstructural analysis of films was performed by SEM. Micrographs of kefiran films without microorganisms or containing both, lactobacilli and yeast, are shown on top of Fig. 1. For all kefiran films a relatively compact structure was observed, however, the film surface was less uniform when the microorganisms were included. Prints of bacteria and yeasts, according to their morphology, were observed in the surface of K-Mix and K_{gly}-Mix films (Fig. 1b and d).

The surface structure of the films was also analyzed by AFM. Images of films without microorganisms and containing a mix of bacteria and yeasts are presented at the bottom of Fig. 1. All evaluated films showed good structural integrity. Calculated roughness parameters are presented in Table 1. Kefiran films, containing microorganisms or not, had roughness over the same range as that of other neutral polysaccharide edible films like hydroxypropyl-methylcellulose or starch (Jiménez, Fabra, Talens, & Chiralt, 2010; Jiménez, Fabra, Talens & Chiralt, 2012). Unplasticized kefiran films were the smoothest and accordingly have the lowest values of roughness parameters. The lactobacilli inclusion did not increase the roughness whereas the presence of yeast in the films did. When glycerol was included as plasticizer a higher roughness value was observed in concordance to the observation performed by Ghasemlou, Khodaiyan, and Oromiehie (2011). On the other hand, the inclusion of the mix of bacteria and yeasts to plasticized films did not increase significantly the roughness parameters (Table 1).

The mechanical characterization of films is presented in Fig. 2. Kefiran films showed tensile strength ranging between 8.1 MPa and 14.6 MPa (Fig. 2a). The incorporation of microorganisms to

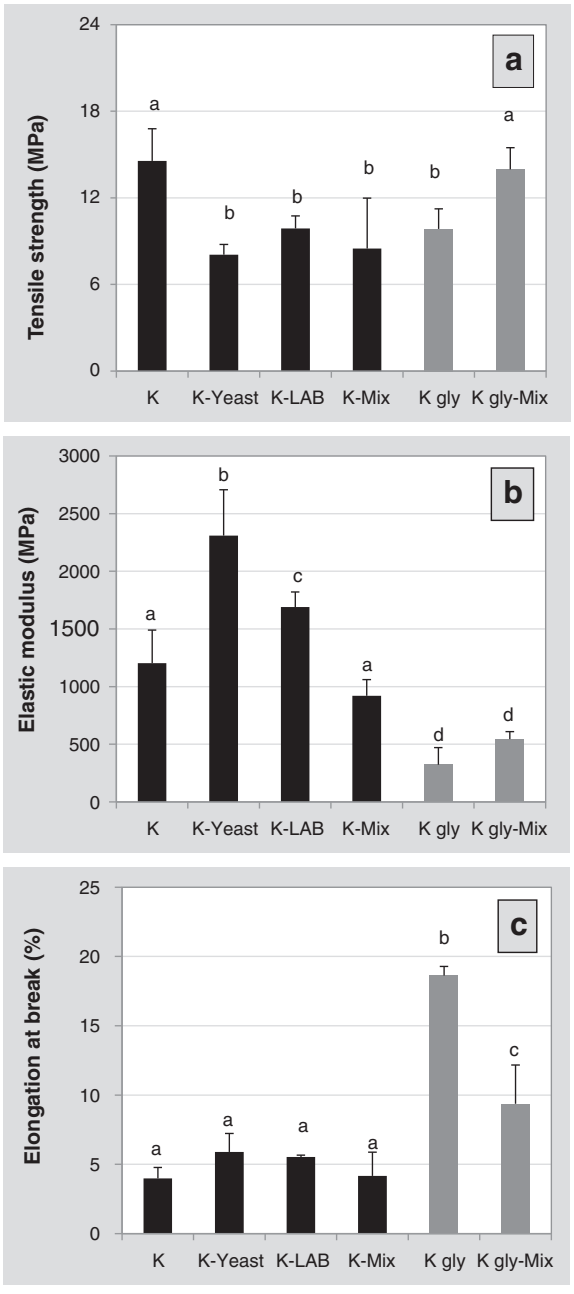

Fig. 2. Tensile strength (a), elastic modulus (b) and elongation-at-break (c) of kefiran films (black bars) or kefiran plasticized with glycerol (gray bars). Kefiran films (K) or kefiran films containing yeast (K-Yeast), lactic acid bacteria (K-Lab) or both microorganisms (K-Mix). Different letters indicate significantly different values with $p \leq 0.05$.

unplasticized kefiran films leads to a significant decrease in this parameter value. The same trend was found by Kanmani and Lim (2013) for pullulan-starch films. Probably the presence of microorganisms causes discontinuities in the matrix and, as a consequence, less intense forces were necessary to break the films. Conversely, when yeast and lactobacilli were included in glycerol plasticized kefiran films (K_{gly}-Mix films) a higher tensile strength was observed indicating that microorganism inclusion could disturb the plasticizing effect of glycerol.

Elastic modulus that varied between 323.3 MPa and 2308.7 MPa (Fig. 2b) were in similar range that the corresponding to the other polysaccharide films containing probiotics (Kanmani & Lim, 2013; Sánchez-González, Quintero Saavedra, & Chiralt, 2013, 2014). In unplasticized kefiran films the individual inclusion of lactobacilli or yeast leads to an increase in this parameter, while the presence simultaneously of both microorganisms did not modify it. In glycerol plasticized films the inclusion of both, lactobacilli and yeast, did not change the elastic modulus value. A similar trend was observed by Sánchez-González et al. (2014) for the incorporation of *Lactobacillus acidophilus* in glycerol plasticized sodium caseinate films.

The films showed elongation-at-break typical of biopolymer films, with values ranging between 4.0% and 18.6% (Fig. 2c). For unplasticized kefiran films, the presence of lactobacilli and yeasts individually or simultaneously did not significantly modify the low elongation values. Glycerol presence significantly increases the elongation of kefiran films, as was reported in previous studies (Piermaria et al., 2009, Piermaria, Bosch, Pinotti, Yantorno, García & Abraham, 2011). In the glycerol plasticized matrix, the presence of microorganisms leads to significantly lower elongation values. The elongation decrease, as a consequence of microbial incorporations, was also observed by Kanmani and Lim (2013) in pullulan films. In contrast, no change in this parameter was reported by Sánchez-González et al. (2013, 2014) for methylcellulose and hydroxypropylmethylcellulose films. The different behavior observed when microorganisms were included to films could be related to the film thickness. Since kefiran and pullulan films were thinner than methylcellulose and hydroxypropylmethylcellulose, the size of included microorganisms would affect the elongation of thin films. Despite this fact, the incorporation of glycerol in kefiran films containing microorganisms (K_{gly}-Mix) substantially improves the elongation in relation with the counterpart unplasticized film (K-Mix).

3.2. Survival of microorganisms included into kefiran films

The film drying step can affect the viability of microorganisms by temperature or oxidative stress, capillary forces, increase in intracellular pH and salt concentration (Fu & Chen, 2011), so maintaining the microorganism's viability throughout the process of production and the subsequent storage of the films was analyzed.

The concentration of viable cells in film-forming dispersions and in the obtained films is shown in Fig. 3. For *K. marxianus* CIDCA 8154 there was no significant decrease in viability during the drying process, regardless of the film type in which this microorganism was included. Conversely, the viability of *L. plantarum* CIDCA 8327 decreased significantly during film obtaining. The same trend was found when the studied microorganisms were dried as free cells from PBS suspensions (data not shown). Cytoplasmatic membrane damage may be responsible for dehydration inactivation since the temperature of drying is not so high as to produce denaturation of critical cell components (Fu & Chen, 2011). Despite the kefiran matrix seemingly not capable of conferring protection against dehydration damage, the survival of lactobacilli in kefiran films was highly satisfactory (63% in K_{gly}-Mix films). This value was comparable with the corresponding *Lactobacillus rhamnosus* GG in sodium alginate films (Soukoulis, Yonekura, Gan, Behboudi-Jobbehdar, Parmenter & Fisk, 2014) or to *L. plantarum* CIDCA 83114 in methylcellulose-based films containing fructo-oligosaccharides (Romano et al., 2014).

The viability of *K. marxianus* CIDCA 8154 and *L. plantarum* CIDCA 8327 included in kefiran films was assessed throughout storage at 20°°C and 75% RH during 60 days (Fig. 4). The viability of *K. marxianus* CIDCA 8154 was maintained throughout the studied storage period and more than 90% of yeasts survived after 30 days of storage at 20°°C. Comparing the three formulated films containing this yeast there were no significant differences in the viability maintenance (Fig. 4a).

On the other hand, the loss of viability of *L. plantarum* CIDCA 8327 depended on the film in which this microorganism was included (Fig. 4b). When this microorganism was incorporated alone in kefiran films its viability decreased sharply and no viable cells were detected after 30 days of storage. When both lactobacilli and yeast were included in the same kefiran films an improvement in the *L. plantarum* CIDCA

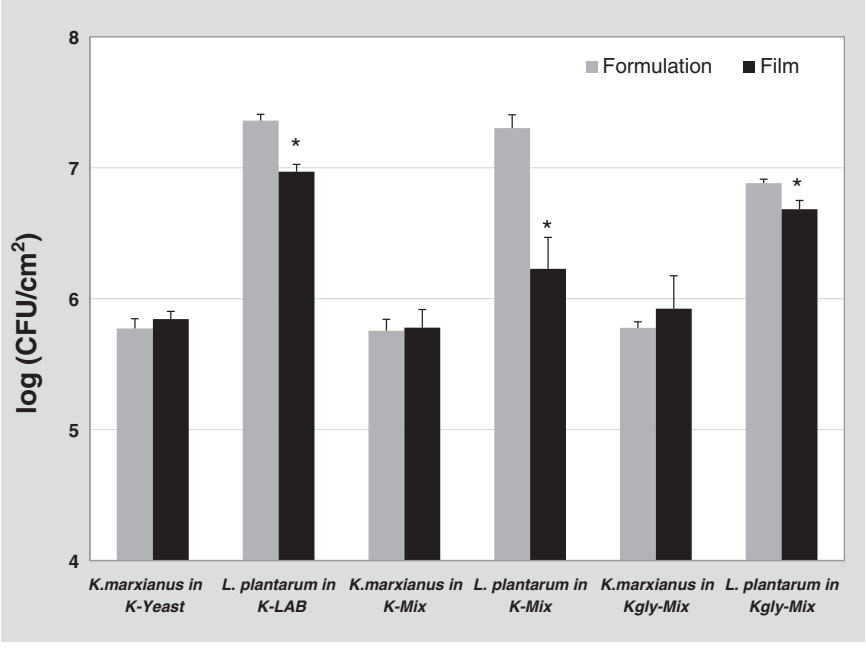

Fig. 3. Survival of *K. marxianus* CIDCA 8154 and *L. plantarum* CIDCA 8327 throughout drying at 37°°C for film obtaining. The numbers of viable microorganisms in film forming dispersion are presented in light gray and the corresponding number in obtained films in black. *Indicates significant differences ($p \leq 0.05$).

8327 survival was observed. The glycerol incorporation in the matrix of films had a positive effect on the survival of these potentially probiotic lactobacilli. In glycerol plasticized kefiran films containing both microorganisms the concentration of *L. plantarum* CIDCA 8327 viable cells descends only two logarithmic cycles after 60 days at 20°°C. This survival was similar to the one reported by Romano et al. (2014) who stored methylcellulose films containing another strain of *L. plantarum* at 4°°C. It is noteworthy that the maintenance of viability of microorganisms in kefiran film was assessed during storage a 20°°C which is an advantage for lactic acid bacteria preservation.

Better growth and survivability during food manufacturing and storage as well as acid and bile resistance could be considered as important factors in maintaining probiotic efficacy (Ranadheera, Baines, & Adams, 2010). The acid, bile and sequential acid–bile tolerance of yeasts and lactobacilli, as free cells or included in kefiran films, was evaluated. The viability decrease as a consequence of the different treatments is presented in Table 2. Survival of both microorganisms exposed to the mentioned stress conditions, presented a different behavior.

L. plantarum CIDCA 8327 free cells decrease (5.86 ± 0.13) logarithmic cycles after acid exposure, however this strain was highly resistant to bile treatment and no changes were observed in the number of viable cells after one hour of bile exposure. For acid–bile sequential treatment, a high decrease in the number of *L. plantarum* CIDCA 8327 viable cells was observed. This value did not differ from the one observed after acid treatment, indicating that the acid is responsible for cell death. The inclusion of *L. plantarum* CIDCA 8327 in kefiran films was able to provide some protection to acid damage, since the diminution of viability after acid treatment was (4.98 ± 0.55) logarithmic cycles. Similarly,

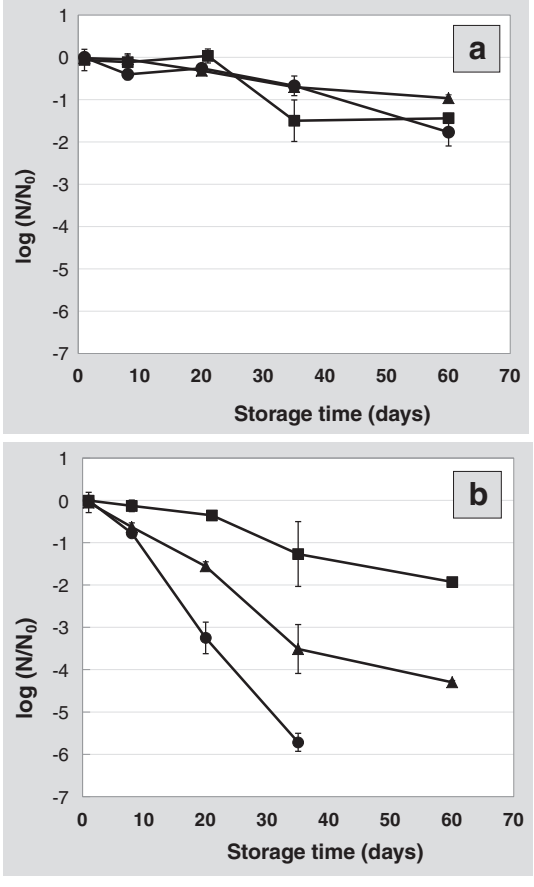

Fig. 4. Survival of *K. marxianus* CIDCA 8154 (a) and *L. plantarum* CIDCA 8327 (b) during film storage at 20°°C. Kefiran films containing individual microorganisms (●), Mix kefiran films (▲) and glycerol plasticized Mix kefiran films (■). Viable microorganisms during storage (N) and in recently obtained film (N_0).

Table 2
Viability decrease of free microorganisms or microorganisms included in glycerol plasticized kefiran films after acid, bile or sequential acid–bile treatment.

Microorganism	Treatment	Viability decrease* (logarithmic cycles)	
		Free cells	Included in K_{gly}-Mix films
K. marxianus CIDCA 8154	Acid	0.43 ± 0.13[a]	0.04 ± 0.08[a]
	Bile	0.09 ± 0.10[a]	0.03 ± 0.09[a]
	Sequential acid–bile	1.06 ± 0.40[b]	0.44 ± 0.02[a]
	PBS	0.00 ± 0.07[a]	0.09 ± 0.11[a]
L. plantarum CIDCA 8327	Acid	5.86 ± 0.13[c]	4.98 ± 0.55[d]
	Bile	0.04 ± 0.06[a]	0.07 ± 0.07[a]
	Sequential acid–bile	5.98 ± 0.24[c]	5.72 ± 0.56[c]
	PBS	0.00 ± 0.05[a]	0.01 ± 0.10[a]

Values are given as mean ± standard deviation. Different letters indicate significant differences ($p \leq 0.05$).

De Barros, Scherer, Charalampopoulos, Khutoryanskiy, and Edwards (2014) found protection of probiotic *Bifidobacterium breve* NCIMB 8807 to acid treatment using polymer matrix containing ethyl cellulose. However no differences in cell viability were observed after sequential treatment indicating that the kefiran film did not exert protection to these microorganisms.

Free cells of *K. marxianus* CIDCA 8154 were highly resistant to acid and bile conditions. Nevertheless, the acid–bile sequential treatment significantly decreased their number. The process of film obtaining did not change the resistance of this yeast to acid or bile media and kefiran film inclusion improved *K. marxianus* CIDCA 8154 survival to the sequential acid–bile media. Probably the initial acid exposition of free cells induces a bile sensibility which is less evident when microorganisms were included in the matrix of kefiran films.

4. Conclusions

Physicochemical characteristic of kefiran films as well as survival of microorganisms during film obtaining indicates that the developed films are adequate carriers for the studied microorganisms. All films presented a good structural integrity. The presence of microorganisms in the matrix, especially yeast, led to a greater variability in mechanical parameters and to a decrease in tensile strength and in extensibility of films. No loss on yeast viability was detected and lactobacillus concentration decreased only one logarithmic cycle during drying stress taking place during film formation. It is noteworthy that evaluated microorganisms survive in non-refrigerated conditions included in kefiran films and have the same or improved resistance to gastrointestinal conditions. Thereby, kefiran matrix with health promoting properties results appropriate for probiotic administration to be used for the prevention of gastrointestinal disorders.

As potentially probiotic microorganisms included in the kefiran film survive for a reasonable period of storage in non-refrigerated conditions in an edible matrix constituted by a bioactive carbohydrate, the proposed films are suitable for application as alternative ways for delivery or storage of probiotics for the development of novel functional products.

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