Innovative Food Science and Emerging Technologies 88 (2023) 103452

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

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

Sustainable starch-based edible films with agrifood residues as potential carriers for the probiotic *Lactobacillus rhamnosus*

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ARTICLE INFO

Keywords:
Edible films
L. rhamnosus
Orange peel
Quince peel
Potato peel

ABSTRACT

Edible films are promising carriers for probiotics and can be composed by agrifood residues, which are usually rich in polymers and bioactive compounds. In this work, starch-based films were enriched with three types of agrifood residues (quince, potato and orange peels) and the incorporation of the probiotic *Lactobacillus rhamnosus* was studied, as well as the addition of inulin as a protective prebiotic. The resulting films were characterized in terms of mechanical properties, physicochemical properties, lactobacilli viability and microbiological properties. The mechanical properties of the films generally decreased with the introduction of L. *rhamnosus*, although this was highly dependent on the film composition. All films exhibited water vapor permeabilities in the typical range of starch-based films and were not greatly affected by the inclusion of probiotics. The loss of probiotic viability during films production was strongly related to the pH of the film-forming solutions. Films with agrifood residues had a slower loss of probiotic viability during storage, when compared to plain starch films, which may be explained by the presence of antioxidant compounds. Inulin was expected to improve viability, but this was not observed. Microbiological analysis showed that agrifood residues powders contained natural contaminant bacteria that were partially eliminated during film formation. Moreover, none of the target foodborne pathogens were detected in the analyzed samples. Overall, the results suggest that edible films containing agrifood residues can be a promising material for the delivery of probiotics and/or as primary packaging for some food products.

1. Introduction

Edible films are thin layers of food grade materials that are applied to food products and that can improve food quality and shelf life by reducing the mass transfer rate of water vapor, gases and volatile compounds between the food and the environment (Mohamed, El-Sakhawy, & El-Sakhawy, 2020; Pop et al., 2020). These protective layers are formed by biopolymers (polysaccharides, proteins) and/or lipids (Mohamed et al., 2020) and a plasticizer, usually glycerol, is also added to improve the flexibility of the films. The addition of bioactive compounds (such as antioxidants, antimicrobials, vitamins, etc) gives edible films the ability to actively preserve the food product and potentially add them nutritional and functional value (Espitia, Batista, Azeredo, & Otoni, 2016; Garcia-Henao et al., 2023). In this context, the incorporation of probiotics in edible films has been proposed and investigated in the last decade (Espitia et al., 2016; Guimarães, Abrunhosa, Pastrana, & Cerqueira, 2018; Pop et al., 2020).

Probiotics are live microorganisms that have a positive impact on human health, namely by improving gut function, when ingested in adequate quantities [a minimum of 10^8–10^9 CFU per day is usually referred in the literature (Espitia et al., 2016; Pop et al., 2020; Sáez-Orviz, Rendueles, & Díaz, 2023)]. The use of edible films as a delivery system for probiotics offers several advantages, such as: i) the delivery of a high dose in a relatively compact volume; ii) the reduction of viability loss during storage, and iii) the protection against the harsh conditions of the upper gastrointestinal tract (Espitia et al., 2016; Pop et al., 2020).

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https://doi.org/10.1016/j.ifset.2023.103452
Received 29 June 2023; Received in revised form 6 August 2023; Accepted 11 August 2023
Available online 12 August 2023

In addition, probiotics incorporated in edible films can protect food products from pathogenic and food spoilage microorganisms by limiting their growth through competition and/or by producing molecules with effective antimicrobial activity (Espitia et al., 2016; Guimarães et al., 2018; Sánchez-González, Quintero Saavedra, & Chiralt, 2013). This protective effect has been reported in a variety of foods, including fresh and minimally processed fruits, (de Oliveira, Fernandes, & de Souza, 2021) and meat and fish products (Guimarães et al., 2018; Mozaffarzogh, Misaghi, Shahbazi, & Kamkar, 2020).

Several probiotics, mainly from the genera *Lactobacillus* and *Bifidobacterium*, have been incorporated into edible films consisting of polysaccharides (e.g., pectin, alginate, carboxymethylcellulose, starch), proteins (e.g., gelatine whey protein, sodium caseinate), or mixtures of both (Espitia et al., 2016; Guimarães et al., 2018; Pop et al., 2020; Sáez-Orviz et al., 2023). The addition of prebiotics such as inulin and fructo-oligosaccharides has also been investigated (Sáez-Orviz et al., 2023). Research has been focused on the development of edible films that can effectively preserve the viability of probiotics. This has involved the screening of film-forming materials that favor the preservation of viability, not only during the film forming process but also during storage (de Oliveira et al., 2021). In parallel, the impact of probiotics incorporation on the barrier and mechanical properties of the films and on their visual aspect (color and transparency) is currently a subject of intense research (Sáez-Orviz et al., 2023).

The use of plant residues from the agrifood processing industry - such as peels, skins, husks, seeds, and pomace - for the production of biodegradable packaging materials, including edible films, is an emerging research area (Karimi Sani et al., 2023; Santhosh, Nath, & Sarkar, 2021), which is aligned with the circular economy and sustainability concepts. These residues are composed of lignocellulosic materials, polysaccharides such as pectin and starch, sugars (mono- and disaccharides), small amounts of proteins and lipids, micronutrients (vitamins and minerals), and a variety of phytocompounds with bioactive properties (Karimi Sani et al., 2023). Alone or in combination with refined film-forming biopolymers (extracted from other plants, animals, microorganisms or algae), these residues can be used to produce films with improved mechanical and barrier properties, functional properties (e.g antioxidant and antimicrobial activity) and with high nutritional value (Karimi Sani et al., 2023; Santhosh et al., 2021).

In a recent work, the authors developed edible films based on potato peel, orange peel and/or quince peel powders combined with potato starch as potential coatings for the preservation of cured cheese (Sebastião, 2021). In the present work, the probiotic *Lactobacillus rhamnosus* was incorporated into these films and its effect on the water vapor barrier properties, mechanical properties, and visual properties of the films was investigated, as well as the effect of each film formulation on the viability of the probiotics during the film forming process. The addition of inulin, a prebiotic frequently used to protect probiotics during drying processes and storage time (Sáez-Orviz et al., 2023) was also tested. To the best of our knowledge, the incorporation of probiotics into such complex edible film matrixes (i.e. containing agro-industrial food residues) has not yet been reported in the literature.

2. Materials and methods

2.1. Materials

Lactobacillus rhamnosus DSM 20021 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, GmbH). MRS broth medium was purchased from Scharlab and bacteriological agar from Biolife. Potato starch was supplied by Sigma-Aldrich and inulin (FRUTAFIT®TEX) by Brenntag Quimica S.A.U. Quinces, potatoes, and oranges were obtained from local producers (Leiria, Portugal). Oranges were peeled manually, and quinces and potatoes were peeled with a knife. Peels were rinsed with 0.1% solution of sodium hypochlorite in water, cut into small pieces, and freeze-dried. Dried peels were crushed with a domestic knife mill and the resulting powders were fractionated by size using a set of sieves. Powders with a granulometry lower than 250 μm were used for the preparation of the films.

2.2. Lactobacillus rhamnosus culture

Lactobacillus rhamnosus was stored at −80 °C in MRS (DeMan, Rogosa and Sharpe) medium supplemented with 15% glycerol. After culturing on MRS Agar medium at 35 °C, few colonies of L. *rhamnosus* were picked and transferred into 45 mL of MRS broth in a sterile plastic conical flask and allowed to growth overnight (16–17 h) in an orbital shaker (100 rpm) at 35 °C. Cells were centrifuged at 5000 ×*g* and, after discarding the supernatant, the biomass was washed twice with phosphate buffered saline (PBS) and resuspended in 45 mL of the film-forming solutions to obtain a cell concentration of about 10^8–10^9 CFU/mL.

2.3. Preparation of probiotic edible films

Films were prepared by solvent casting. Eight different formulations combining starch (S), potato peel (P), quince peel (Q), orange peel (O) and inulin (In) were investigated. Glycerol was used as plasticizer. The detailed composition of the formulations is described in Table 1.

Film-forming solutions were prepared by dissolving potato starch and glycerol in 100 mL of sterile boiling water in a round-bottom glass flask. Inulin was added, if used, and powders from agrifood residues were dispersed in the solutions. The flasks were stirred at 100 rpm for one hour while immersed in a water bath at 95 °C. The solutions were filtered through an 80 μm nylon mesh to remove undissolved large particles and allowed to cool to approximately 35 °C. Finally, and after measuring the pH of the solutions, the previously prepared biomass of L. *rhamnosus* was added and homogenized by vortexing.

Probiotic films were prepared by pouring 15 mL of film-forming solutions containing homogeneously suspended biomass into sterile 90 mm Petri dishes, which were left to dry overnight at 35 °C in a forced-air oven. For probiotic enumeration assays and films microbiological characterization smaller films were prepared by casting 6 mL of film-forming solutions into 47 mm sterile Petri dishes. In parallel, and as controls, films of the same formulations but without added probiotics were also prepared.

After drying, the films were peeled off and stored until further use at room temperature and ~ 53% relative humidity (RH), in a desiccator containing a saturated magnesium nitrate solution. All procedures of probiotic growth and incorporation were performed within a BSL-2 laminar flow chamber.

2.4. Films characterization

2.4.1. Thickness and color

Films thickness was measured with a digital micrometer with an accuracy of 0.001 mm. Five measurements were taken from five random

Table 1
Composition of the film forming solutions.

Film Label	Film Formulations (g/100 mL)					
	Starch (S)	Glycerol	Quince Peel (Q)	Orange Peel (O)	Potato Peel (P)	Inulin (In)
S	2.0	0.4	–	–	–	–
S-In	2.0	0.4	–	–	–	0.4
SQ	2.0	0.4	0.4	–	–	–
SQ-In	2.0	0.4	0.4	–	–	0.4
SO	2.0	0.4	–	0.4	–	–
SO-In	2.0	0.4	–	0.4	–	0.4
SPQ	1.0	0.4	0.4	–	1.25	–
SPQ-In	1.0	0.4	0.4	–	1.25	0.4

zones and used to calculate the average thickness.

The color of the films was evaluated with an NH310 *High-Quality CIE (International Commission on Illumination) and CNS (China National Standards)* colorimeter. Readings of lightness, L* (0 = black to 100 = white), and chromaticity coordinates, a* (−60 = green to +60 = red) and b* (−60 = blue to +60 = yellow), were taken at six different points. The total color difference between films with lactobacillus and plain films was calculated according to the formula:

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

2.4.2. Mechanical properties

Films tensile properties were measured using a texturometer (Stable microsystems TA XT Express, UK), working at a crosshead speed of 1.0 $mm.min^{-1}$. The films were cut into dumbbell-shaped test pieces with 60 mm length, 10 mm width at the ends and 5 mm width at the narrow portion, according to norm ISO 527-2 (ISO, 2012) and conditioned at appx. 53% RH in a desiccator containing a saturated magnesium nitrate solution until testing. Maximum tensile strength (MPa), tensile strain at break (%), and Young's modulus (GPa) were calculated by the instrument software. At least four measurements were made for each type of film.

2.4.3. Water vapor permeability

The water vapor permeability (WVP) of the films, with and without L. *rhamnosus*, was determined by a gravimetric method, according to the norm ASTM E96 (ASTM, 1995). Film samples were sealed over a circular opening of a glass cell filled up to approx. 2/3 of its height with anhydrous calcium chloride, previously dried at 200 °C. The glass cells were then transferred to a growth chamber (Binder, model KBWF 240) at 25 °C and 80% RH, and at predetermined times the glass cells were removed from the chamber and immediately weighed. WVP was calculated using the following equation:

$$WVP = \frac{G}{A \times \Delta RH \times P_{W}} \times \delta$$

Where: δ (m), is the average film thickness; G (g/s), the permeation rate, obtained from the linear regression of the experimentally recorded mass versus time; A (6.36×10^{-5} m^2), the permeation area of the films; ΔRH (0.80), the difference between RH outside and inside the permeation cell; and, P_w, the partial water vapor pressure at the test temperature (3.168×10^{3} Pa). Determinations were performed in triplicate for each film type.

2.5. Viability of L. rhamnosus

The viability of L. *rhamnosus* in the film-forming solutions and formed films was evaluated by a plate count method. For film-forming solutions, serial dilutions were prepared with PBS and the appropriate dilutions were inoculated and spread on MRS agar Petri dishes. Petri dishes were then incubated at 35 °C for 48 h before colony counting. Plates were prepared in triplicate for each dilution.

The viability of L. *rhamnosus* incorporated in the formed films was evaluated by dissolving an entire film (formed in a 47 mm diameter Petri dish) in 6 mL of PBS (corresponding to the same volume of the film forming solutions). To achieve complete disintegration of the films, the film solutions were processed with an IKA T18 Ultra-Turrax at approx. 20000 rpm for 10 s, followed by one hour in an orbital shaker at 35 °C and 100 rpm. Solutions were then serially diluted with PBS, plated onto MRS agar plates, and incubated at 35 °C for 48 h before plate counting. Two films were prepared for each formulation and MRS agar plates were prepared in duplicate for each film.

L. rhamnosus viability during films storage was accessed for films conditioned at 5 ± 3 °C and a RH of appx. 65%. At predetermined times, two films for each formulation were removed from storage and processed as described above. Loss of viability was expressed as Log N/N_0, where N_0 is the number of viable bacteria in the films at the beginning of the assay and, N, is the number of viable bacteria at predetermined times, expressed as CFU/mL.

2.6. Microbiological profile of films and raw materials

To detect the presence of natural contaminant foodborne pathogens in the films obtained from different formulations, a protocol adapted from Bento, Alarico, Empadinhas, de Sousa, and Braga (2022) was followed and minor adjustments were performed. All procedures were conducted within a BSL-2 laminar flow chamber. First, film samples were placed in sterile 50 mL Falcon tubes, and a suspension of each sample was prepared by adding 45 mL of Brain Heart Infusion broth (BHI) diluted 5-fold, followed by gentle agitation (100 rpm) in an orbital shaking incubator at 35 °C for 30 min, to maximize bacterial recovery. As a control, a tube containing only the diluted BHI medium was subjected to the same treatment. A portion (5 mL) of each suspension was filtered (0.22 μm pore size, Millipore, Merck) using an EZ-Fit™ Manifold system and placed into different culture media. All the plates were incubated at 35 °C for 20 days and monitored regularly for detection and enumeration of emergent colonies to assess the level of contamination of film samples – semi-quantitative assessment (S-QA). Three nonselective rich media, namely, Yeast Extract-Peptone-Dextrose (YPD) Agar, Tryp Soy Agar (TSA), and Chocolate Agar (ChocA) were used, respectively, to detect the growth of yeast/fungi, general bacteria, and fastidious bacteria. For selective detection of specific foodborne pathogens, the following media were used: Mannitol Salt Agar (MSA) to detect *Staphylococcus aureus*, CHROMagar Listeria, CHROMagar Salmonella and CHROMagar Campylobacter media to detect *Listeria* spp., *Salmonella* spp. and *Campylobacter* spp., respectively. The Reinforced Clostridial (RC) medium was used to detect *Clostridium* spp. and other anaerobes. These plates were incubated at the same temperature but inside an appropriate chamber with a CO_2 atmosphere. For the analysis of film samples prepared with incorporated L. *rhamnosus*, also 5 mL of the dissolved films solutions were filtered and plated into MRS Agar medium. The components used in films formulations (Table 1), glycerol, commercial starch, inulin, and powders from quince, potato, and orange peels, were also subjected to the same microbiological analyses.

2.7. Statistical analysis

Results are presented as mean ± standard deviation. Multiple groups were compared by one-way ANOVA followed by post hoc Tukey test (SigmaPlot 12.0). Comparisons between two groups were made by two-tailed Student's *t*-test (graphpad.com/quickcalcs). A p-value <0.05 was considered statically significant.

3. Results and discussion

The films thickness, presented in Table 2, ranged from 30 to 60 μm and, as expected, values increased with the increase in total solids in the film-forming solutions (see Table 1). Overall, the films with probiotics were slightly thicker, although these differences were statistically significant ($p < 0.05$) only for a few formulations (S vs *L.rh* S, SQ vs *L.rh* SQ and SO vs *L.rh* SO). These results are consistent with those reported in the literature, where no difference in thickness or a slight increase in thickness is usually reported as a result of the incorporation of probiotics into the films (Ebrahimi et al., 2018; Lee, Yusof, & Pui, 2020; Nisar et al., 2022; Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, & Fisk, 2014).

The color parameters of the films with and without lactobacilli are also included in Table 2. Considering only the films without probiotics, the greatest color differences were observed between the films composed only of starch (S and S—In) and the films partially formed from agrifood residues (SQ, SQ-In, SO, SO-In, SPQ, and SPQ—In). While

Table 2
Color parameters and thickness of the films with and without *L. rhamnosus*.

Films Code	Color Parameters				Thickness (μm)
	L*	a*	b*	ΔE^*	
S	*96.94 ± 0.27*	*−0.21 ± 0.05*	*0.47 ± 0.17*		*31 ± 2*
L.rh S	*96.73 ± 0.10*	*−0.28 ± 0.02*	*1.82 ± 0.17*	1.37	*35 ± 3*
S-In	*97.19 ± 0.09*	*−0.11 ± 0.03*	*0.30 ± 0.06*		39 ± 4
L.rh S-In	*96.70 ± 0.26*	*−0.23 ± 0.06*	*1.54 ± 0.62*	1.34	41 ± 2
SQ	91.52 ± 0.64	2.29 ± 0.47	*8.38 ± 0.83*		*46 ± 4*
L.rh SQ	90.86 ± 0.56	2.32 ± 0.33	*10.73 ± 0.88*	2.44	*56 ± 5*
SQ-In	*93.10 ± 0.28*	*1.01 ± 0.15*	*6.76 ± 0.41*		54 ± 4
L.rh SQ-In	*88.44 ± 1.00*	*4.15 ± 0.68*	*13.53 ± 1.10*	8.80	58 ± 6
SO	95.25 ± 0.23	*−0.54 ± 0.04*	6.09 ± 0.35		*40 ± 6*
L.rh SO	95.16 ± 0.16	*−0.42 ± 0.04*	6.22 ± 0.37	0.20	*56 ± 3*
SO-In	95.28 ± 0.05	−0.50 ± 0.01	5.99 ± 0.14		54 ± 5
L.rh SO-In	95.22 ± 0.19	−0.47 ± 0.05	6.32 ± 0.51	0.34	55 ± 7
SPQ	*86.25 ± 0.34*	*3.65 ± 0.14*	*13.77 ± 0.27*		59 ± 6
L.rh SPQ	*88.31 ± 0.44*	*2.61 ± 0.21*	*10.65 ± 0.46*	3.88	52 ± 4
SPQ-In	*87.58 ± 0.82*	2.81 ± 0.38	10.03 ± 0.79		59 ± 3
L.rh SPQ-In	*88.65 ± 0.62*	2.66 ± 0.35	10.79 ± 0.68	1.32	60 ± 5

* Italicized values indicate statistically significant differences between the properties of the films with and without lactobacillus, for the same film composition.

the former were practically achromatic (i.e., a* and b* parameters close to zero), the latter showed a slight increase in the a* parameter and a relatively high increase in the b* parameter. Films with and without inulin with the same agrifood components had comparable color parameters, indicating that the introduction of inulin had little effect on the color of the films. The addition of quince and potato peels resulted in films with a slight red tint (slightly positive a* values), while the films with orange peel remained with identical a* values of the starch films.

Compared to the S and S—In films, all films with agrifood residues had a yellowish tone (corresponding to higher +b*), especially the films with both quince and potato peels (SPQ and SPQ-In films). The films with agrifood residues were also slightly darker (lower L* values) than the starch films, particularly the SPQ and SPQ-In films.

The differences observed between films with and without lactobacilli for the same formulation were very subtle, as can be seen from the low values of color difference (AE*), almost all of which were below 3, a threshold above which color differences are easily perceived by the human eye (Davachi, Pottackal, Torabi, & Abbaspourrad, 2021). Nevertheless, and even minimally, the prevailing trend due to the introduction of probiotics was a slight increase in yellowness and redness, as described in the literature for other films (Lee et al., 2020).

The mechanical properties of the films are shown in Fig. 1. Considering only the films without probiotics, and comparing the films with peels powders (SQ, SQ-In, SO, SO-In, SPQ and SPQ—In) with the films composed only of starch and glycerol or starch, glycerol and inulin (S and S—In), the latter had the highest values of tensile strength (14.4 and 21.8 MPa, Fig. 1a) and Young's modulus (0.7 and 1.0 GPa, Fig. 1c), and the lowest values of strain at break (3.2% and 8.6%, Fig. 1b), indicating greater robustness and rigidity. In this case, the addition of inulin, known for its plasticizing effect (Orozco-Parra, Mejía, & Villa, 2020; Sáez-Orviz et al., 2023), did not significantly modify the properties of the S—In film compared to the S film. This may be related to the concentrations used. Films with agrifood residues had significantly lower tensile strengths (1.5–6.9 MPa) than S and S—In films, which were identical to each other ($p > 0.05$). In general, films with agrifood residues also had lower Young's modulus (0.06–0.49 GPa) and higher values of strain at break (10–51%), indicating greater flexibility and extensibility, possibly provided by pectin, which is present in significant amounts in orange peels and quince peels (Ángel Siles López, Li, & Thompson, 2010; Othman et al., 2022), and/or by the plasticizing effect of monosaccharides and disaccharides (Zhang & Han, 2006), of which orange and quince peels are also rich (Ángel Siles López et al., 2010; Othman et al., 2022). For strain at break, the addition of inulin seems to have a synergistic effect, as SQ-In, SO-In and SPQ-In films had the highest values for this property.

The effect of the incorporation of L. *rhamnosus* on the mechanical properties of the films had no obvious trend, depending the effect on the film formulation. For example, for tensile strength, only the S—In, SQ-In, SO and SPQ-In films had significantly different values (compared to the films without lactobacilli) and among them the dominant tendency was a decrease of this parameter, except for the SPQ-In film, for which a slight increase was observed. Similarly, for strain at break and Young's modulus, and among films with significant differences, the dominant trend was for a decrease in these properties with the incorporation of L. *rhamnosus*. Similar results are reported in the literature, where is described that the effect of incorporating probiotics in edible films either does not affect the mechanical properties or generally causes a slight decrease in them, depending on the nature of the film components, the type of microorganism, and the interactions established between them (Sáez-Orviz et al., 2023). For example, Soukoulis, Singh, Macnaughtan, Parmenter, and Fisk (2016) reported that the incorporation of L. *rhamnosus* in starch-protein based films did not alter the mechanical properties of the films, and Davachi et al. (2021) found no significant differences between mucilage base films with and without L. *rhamnosus*. On the other hand, Ebrahimi et al. (2018) reported a decrease in tensile strength and strain at break (EB) with the incorporation of different probiotics, including L. *rhamnosus*, in caboxymethyl cellulose based films. The same trend was observed by Nisar et al. (2022) in citrus pectin based films.

Fig. 2 shows the water vapor permeability (WVP) of the edible films with and without *L. rhamnosus*. Statistically, there were no significant differences in WVP values among films from different formulations ($p > 0.05$), as all of them had WVP values within the range reported in the literature for starch-based films (Agarwal, 2021). In general, the incorporation of probiotics increased the WVP values, although this increase was significant ($p < 0.05$) only for films without agrifood residues (S and S—In), where for the S film, the WVP value increased from 1.1×10^{-10} to 1.7×10^{-10} $g.m^{-1}.s^{-1}.Pa^{-1}$ with the incorporation of the probiotic, and for the S—In film, the WVP increased from 1.0×10^{-10} to 1.9×10^{-10} $g.m^{-1}.s^{-1}.Pa^{-1}$. These results are in line with the literature, where there is a general trend towards an increase in the WVP of edible films with the introduction of probiotics. (Ebrahimi et al., 2018; Nisar et al., 2022; Sánchez-González et al., 2013). This effect is usually explained by the fact that the presence of bacteria introduces discontinuities in the polymer matrix, reducing its compactness and cohesion and thus promoting water permeation (Sáez-Orviz et al., 2023; Sánchez-González et al., 2013).

The loss of probiotic viability during film formation (drying process) can be seen in Fig. 3, which shows the viability of L. *rhamnosus* in the film-forming solutions and in the newly formed films. According to Fu and Chen (2011), the main mechanisms of microorganisms' inactivation/death during a convective drying process are heat-induced or dehydration-induced. Heat damage mainly affects the degradation of essential cellular structures, such as ribosomes, while dehydration damage mainly leads to the rupture of the cytoplasmic membrane due to the osmotic and oxidative stress. Thus, the degree of viability loss during

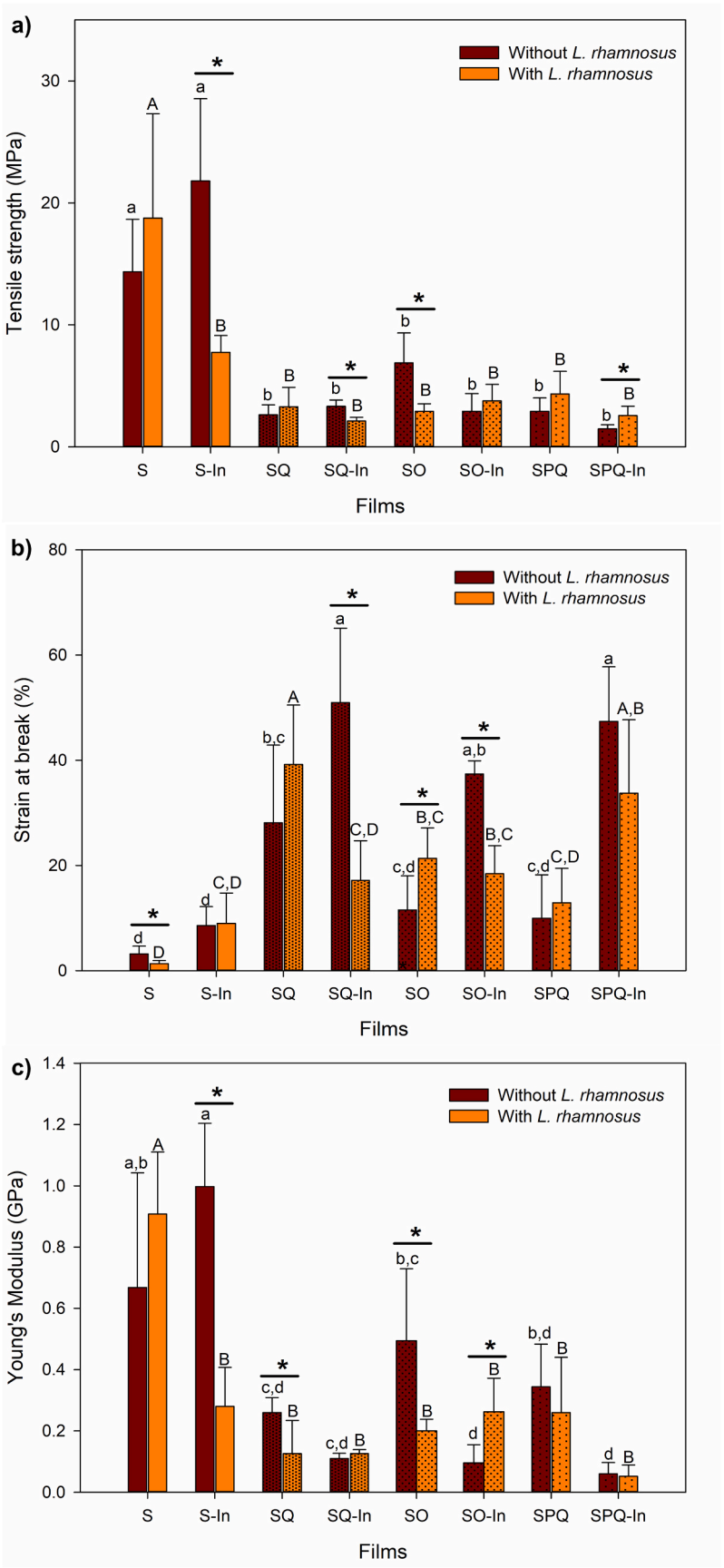

Fig. 1. Mechanical properties of the films, with and without *L. rhamnosus*. a) Tensile strength; b) Strain at break; c) *Young's Modulus*. Different letters indicates statistically significant differences ($p < 0.05$). The symbol (*) indicates that for films with the same formulation there are statistically significant differences between films with and without L. *rhamnosus* ($p < 0.05$).

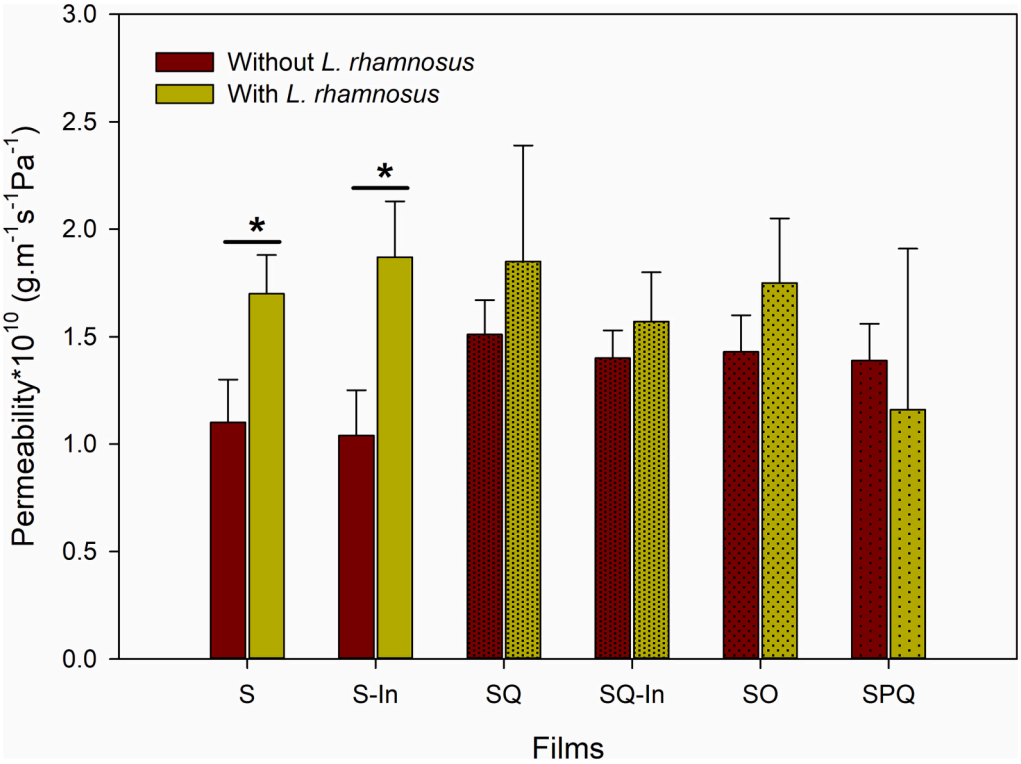

Fig. 2. Water vapor permeability of the films with and without *L. rhamnosus*. No statistically different results were found between the films with L. *rhamnosus* ($p > 0.05$) and among the films without L. *rhamnosus* ($p > 0.05$). The symbol (*) indicates that there are, for the same film formulation, significant differences between films with and without L. *rhamnosus* ($p < 0.05$).

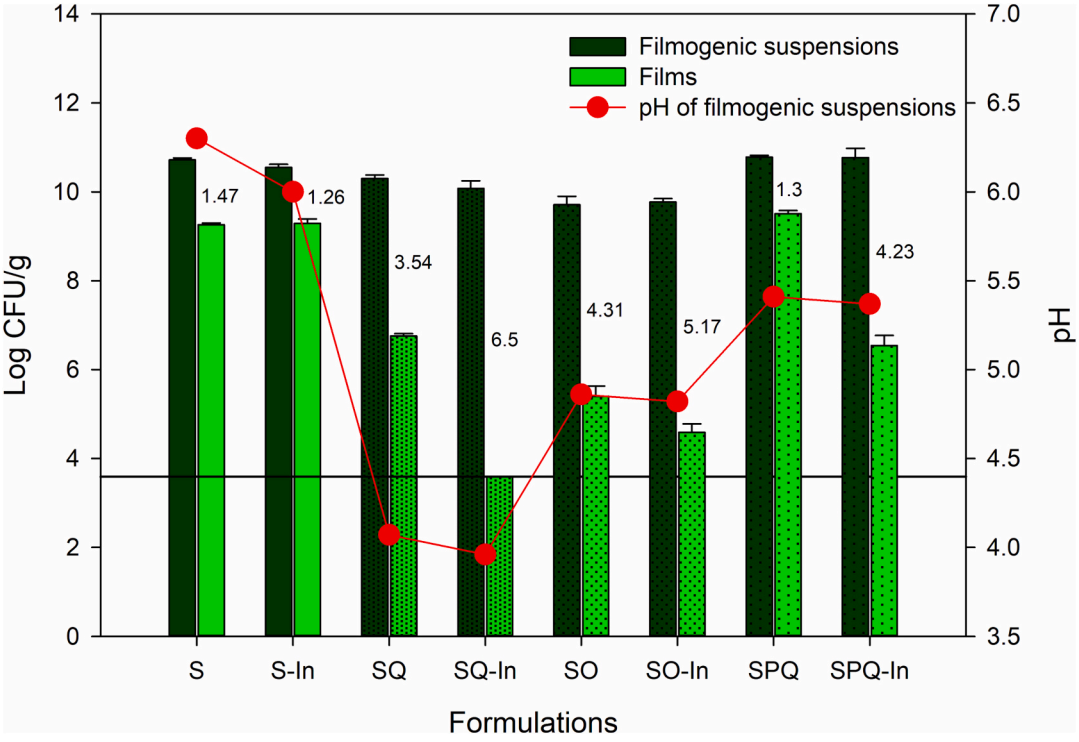

Fig. 3. Left axis: Viability (Log CFU/g) of film-forming solutions and formed films. Right axis: pH of the film-forming solutions before addition of L. *rhamnosus*. Inserted values: Loss of viability. The horizontal line (log 3.6) corresponds to the lowest dilution made for plate count).

a convective drying process is determined by intrinsic factors (i.e. microorganisms tolerance to environmental stress, namely heat stress, and osmotic stress) and extrinsic factors such as drying conditions and the characteristics of the carrier material (Fu & Chen, 2011). Drying conditions, such as drying temperature and drying rate, can be optimized to minimize the heat and osmotic stress and thus maximize the cell

viability. Similarly, the composition of the carrier/encapsulant can be formulated to maximize the protection of the microorganisms.

Considering the low drying temperature of the films (35 °C), the effect of heat stress on the survival rate of L. *rhamnosus* is expected to be low (Soukoulis et al., 2014; Soukoulis et al., 2016; Soukoulis, Behboudi-Jobbehdar, Macnaughtan, Parmenter, & Fisk, 2017).

In this work, viability loss was significant for all films, ranging from 1.26 log CFU/g (for S—In) to values higher than 6.5 log CFU/g (for SQ-In). The viability loss due to the drying process was of 1.47 log CFU/g and 1.26 log CFU/g for S film and S—In film, respectively. These values are similar to those reported by Soukoulis et al. (2016) for L. *rhamnosus* immobilized on starch-based films (1.71 log CFU/g and 1.81 log CFU/g for *L. rhamnosus* incorporated into corn starch and rice starch films, respectively). The same authors also found that the addition of a protein (gelatin, soy protein concentrate or sodium caseinate) to starch resulted in a reduction in viability loss (for values around 1 log CFU/g). In general, protein-based films are more efficient in protecting probiotics from inactivation during drying (de Oliveira et al., 2021), presumably due to the ability of proteins to scavenge free radicals and provide nutrients (peptides and amino acids) (Fu, et al., 2011; Soukoulis et al., 2016).

Apart from the SPQ formulation, all formulations containing agrifood residues showed extreme viability losses (> 3.5 log CFU/g). These results are certainly related to the pH of the initial film-forming solutions, also represented in Fig. 3, where a correlation between the pH of the film-forming solutions and the viability of L. *rhamnosus* in the resulting films can be observed. The film-forming solutions of S and S—In films, the films with the lowest viability losses, had a pH of 6–6.3, a pH within the ideal ranges for L. *rhamnosus* growth (Soukoulis et al., 2017). In contrast, the formulations with quince peel (pH ~ 4.0) and orange peel (pH ~ 4.8) had the lowest pH values and, generally, resulted in films with the highest viability losses. Extreme viability losses (3.06 log CFU/g) due to the low pH of the film-forming solution (pH ~ 4) have been reported by Soukoulis et al. (2017) for *L. rhamnosus* incorporated into citrus pectin films. The addition of P to Q increased the pH of the solutions (pH ~ 5.4), resulting in SPQ films with more favorable viability losses (1.3 log CFU/g), whilst the extreme viability loss of film SPQ-In (4.23 log CFU/g). In fact, and contrary to our expectations, the addition of inulin to the formulations containing agrifood residues resulted in an extreme decrease in viability. This was not observed for the films based only on commercial starch (the S—In film shows a slightly lower viability loss than the S film), which suggests that the combination of inulin with quince and potato powder results in a strong negative effect on probiotics viability, probably due to the resulting overall compositions.

The loss of viability of L. *rhamnosus* in SPQ and S films for one month of storage at approximately 4 °C and 65% RH is represented in Fig. 4. The rate of viability loss of S was so high that at the end of the first week of storage no L. *rhamnosus* colonies were observed on the MRS agar plates inoculated with the lowest dilution. In contrast, the loss of viability of *L. rhamnosus* in SPQ films was more gradual and could be quantified until the end of the study. It is speculated that the inactivation of probiotics during storage is a consequence of the continuation of cellular metabolic processes at a slow rate (Fu, et al., 2011). Thus, storage conditions that allow the free movement of molecules and promote biochemical reactions, such as high temperature, high humidity, and the exposure to air, result in a rapid decrease in cell viability during storage (Fu, et al., 2011). When cells are immobilized in a protective carrier, such as the polymer matrix of an edible film, the physical state of the carrier (glassy or rubbery state) and its ability to permeate water and oxygen are also important (Soukoulis et al., 2014; Soukoulis et al., 2016; Soukoulis et al., 2017). Furthermore, the oxidation of the cytoplasmatic membrane lipids is believed to be the main cause of probiotic inactivation during storage. Therefore, the free radical scavenging capacity of the carrier is a very important factor for probiotics survival. Considering this, the addition of antioxidants to carrier formulations and encapsulation matrices has been suggested (Ying et al., 2011). More recently, the *co*-encapsulation of probiotics and plant extracts in polymeric microparticles has been investigated by several authors. Holkem, Silva, and Favaro-Trindade (2022) reviewed those studies and highlighted the potential of the protective effect of plant extracts rich in phenolic compounds to preserve probiotics exposed to the adverse gastrointestinal conditions and during storage. However, Holkem et al. (2022) also identified the need to optimize the extract composition and

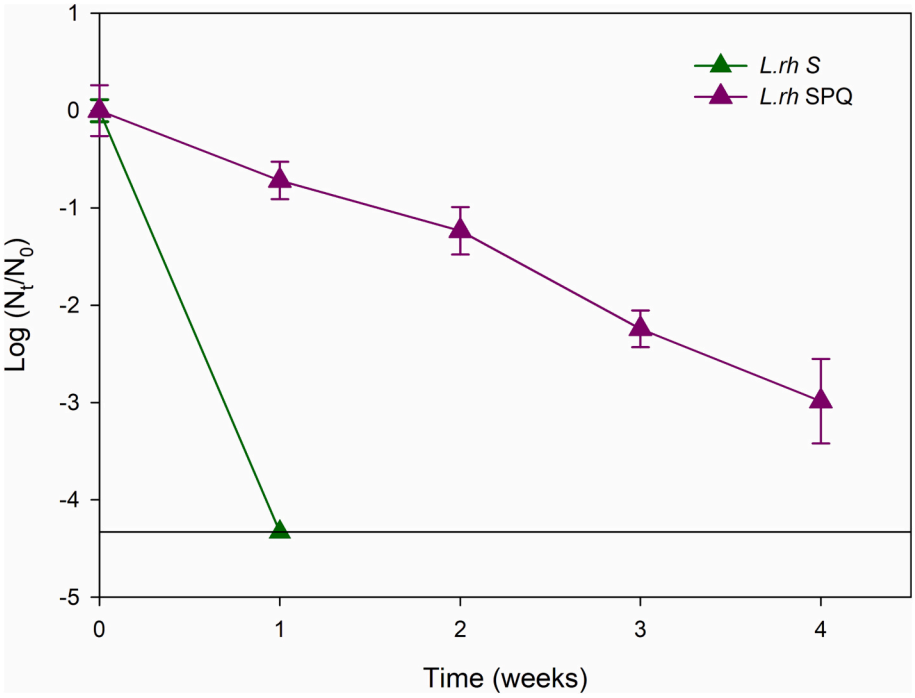

Fig. 4. Loss of viability during storage of L. *rhamnosus* incorporated into S and SPQ films. Storage conditions: 5 ± 3 °C and ~65% RH. The horizontal line indicates that Log N/N0 = − 4.329 (corresponding to the lowest dilution made to plate count).

concentration, as these extracts may also have a potential antimicrobial effect. Considering these findings and the high amount of phenolics and other antioxidant compounds present in the potato peels (Akyol, Riciputi, Capanoglu, Caboni, & Verardo, 2016) and quince peels (Othman et al., 2022) incorporated in the SPQ film, it is reasonable to speculate that the reduced rate of viability loss of L. *rhamnosus* in this film compared to the plain S film is due to the antioxidant protection conferred by the phytochemicals present in the SPQ matrix.

The films were not subjected to any sterilization method, but the initial film-forming solutions underwent a boiling protocol step, which is expected to eliminate most bacteria naturally present in the raw materials. Different selective media were used to detect a wide range of bacteria, including species that are known foodborne pathogens, fastidious bacteria, anaerobic bacteria naturally present in food, and bacteria that could potentially originate from human handling, such as the staphylococci (Kadariya, Smith, & Thapaliya, 2014). Table 3 provides information on the results of this microbiological analysis.

Controls performed as part of each set of microbiological analyses were free of bacteria, as expected, validating the conditions under which the samples were processed. Microbiological analyses were also performed on each component used in the film formulations. The results revealed that the agrifood residues (quince/orange/potato peels) powders contained substantial amounts of natural contaminant bacteria, while the commercial starch and inulin powders, as well as glycerol, were free of contaminants. These results also support the absence of cross-contamination during sample processing. None of the target foodborne pathogens, namely, *Camphylobacter* spp., *Listeria monocytogenes*, or *Salmonella* spp., were detected in the 64 films and 15 film component samples analyzed (Table 3). Using non-selective media, TSA, ChocA and YPD, a high number of bacterial colonies were recovered from the samples, in some cases >50 CFUs, as indicated by the semi-quantitative assessment in Table 3. Probably, the initial boiling steps did not eliminate all the intrinsic bacteria of the agrifood residues. The exception was observed for SO and SO-In films, both with and without L. *rhamnosus*, and for SQ and SQ-In films, also with and without incorporated L. *rhamnosus*. For these films no bacterial contamination was detected in most of the replicates, or in some cases only a single colony was detected. As previously observed in the L. *rhamnosus* viability studies, the significantly low number of colonies is probably related to the low pH of the film-forming solutions of these films (Fig. 3). A similar absence of culturable bacteria was found for these samples in the RC medium (Table 3), indicating that the growth of possible anaerobes initially present in the raw materials was also affected by the final pH of the film formulations. The detection of *Staphylococcus* spp., especially *S. aureus*, which can cause severe food poisoning and outbreaks, is generally performed to determine whether a food is a potential source of food poisoning or to detect post-processing contamination, usually due to human contact with food or food contact with contaminated surfaces (Kadariya et al., 2014). In this study, the selective and differential MSA medium was used to detect staphylococci. In samples with bacterial growth on the MSA medium no color change from pink to yellow was detected around the colonies, indicating the absence of *S. aureus* colonies. Furthermore, bacterial growth on MSA was observed only in the samples of the agrifood residues and films containing these residues, especially films with potato peels (SPQ and SPQ-In films, with or without L. *rhamnosus*). This suggests that these bacteria, at least the Gram-positive bacteria that grew in MSA medium, were naturally present in the agrifood residues powders, but not in the other film components (commercial starch, inulin, and glycerol), nor did sample manipulation appear to be a source of staphylococci. In further studies it will be important to explore additional sterilization methods for agrifood residue powders used in edible probiotic films intended for the food industry. Sterilization of raw materials is crucial as it extends the shelf life of food products, maintains their quality, and promotes food safety, thereby ensuring regulatory compliance (Sruthi & Rao, 2021).

4. Conclusions

The probiotic *Lactobacillus rhamnosus* was incorporated into starch-based films containing different agrifood residues (quince peels,

Table 3
Films and raw materials microbiological results.

Film code (n° of samples)	Culture Agar Media /n° of samples (S-QA)*								
	TSA	ChocA	YPD	Camphyl	Listeria	Salmon	MSA	RC	MRS
Control (10)	0	0	0	0	0	0	0	0	0
S (4)	3(4,++,+++)	4(1,+,++,+++)	2(+),2(+++)	0	0	0	0	4(+++)	–
***L.rh* S** (4)	3(++),1(+++)	3(++),1(+++)	4(++)	0	0	0	1(1)	4(+++)	4(+++)
S-In (4)	3(++),1(+++)	2(++),2(+++)	4(+++)	0	0	0	0	4(+++)	–
***L.rh* S—In** (4)	1(1)	0	0	0	0	0	0	4(+++)	4(+++)
SQ (4)	2(1,+++)	1(+++)	1(+++)	0	0	0	2(+++)	1(+)	–
***L.rh* SQ** (4)	0	0	0	0	0	0	0	0	2(6,7)
SQ-In (4)	1(1)	1(+++)	1(1)	0	0	0	0	0	–
***L.rh* SQ-In** (4)	0	0	0	0	0	0	0	0	1(1)
SO (4)	1(1)	0	0	0	0	0	0	0	–
***L.rh* SO** (4)	0	0	0	0	0	0	0	0	0
SO-In (4)	1(1)	1(1)	1(1)	0	0	0	0	0	–
***L.rh* SO-In** (4)	1(1)	0	0	0	0	0	0	0	0
SPQ (4)	4(+++)	4(+++)	4(+++)	0	0	0	4(+++)	4(+++)	–
***L.rh* SPQ** (4)	4(+++)	4(+++)	4(+++)	0	0	0	4(+++)	4(+++)	4(+++)
SPQ-In (4)	1(+++)	1(+++)	3(+++)	0	0	0	4(+++)	2(+++)	–
***L.rh* SPQ-In** (4)	4(+++)	4(+++)	4(+++)	0	0	0	2(2,4),2(+)	4(+++)	4(+++)
Starch (2)	0	0	0	0	0	0	0	0	–
Glycerol (2)	0	0	1(1)	0	0	0	0	0	–
Quince Peel (3)	3(+++)	3(+++)	3(+++)	0	0	0	3(+++)	3(+++)	–
Orange Peel (3)	3(+++)	3(+++)	3(+++)	0	0	0	3(+++)	3(+++)	–
Potato Peel (3)	3(+++)	3(+++)	3(+++)	0	0	0	3(+++)	3(+++)	–
Inulin (2)	0	0	0	0	0	0	0	0	–

* Number of contaminated samples out of the total samples analyzed along with their corresponding semi-quantitative assessment (S-QA). If the number of colony-forming units (CFUs) was 4 or less, the numerical value was indicated. If the number of CFUs was >4, the following correspondence was adopted: + for 5 to 10 CFUs, ++ for 11 to 50 CFUs, and +++ for >50 CFUs. A numerical value of 0 indicated that no CFUs were detected. Culture Agar media: **TSA** - Tryp Soy Agar; **ChocA** - Chocolate Agar; **YPD** - Yeast Extract-Peptone-Dextrose Agar; **Campyl** - CHROMagar Campylobacter medium; **Listeria** - CHROMagar Listeria medium; **Salmon** - CHROMagar Salmonella medium; **MSA** - Mannitol Salt Agar; **RC** - Reinforced Clostridial medium was used to detect Clostridium spp. and other anaerobes; **MRS** - DeMan, Rogosa and Sharpe Agar medium.

orange peels and potato peels) and inulin as a prebiotic.

The mechanical and physicochemical properties of the films were mainly determined by their composition, while the incorporation of L. *rhamnosus* caused minor changes in the films properties. The loss of probiotic viability during the film forming process was found to be highly dependent on the film composition, which correlated with the pH of the film-forming solutions. Furthermore, and contrary to expectations, inulin had a negative effect on probiotic viability. Evaluation of L. *rhamnosus* viability loss during storage showed that the film with agrifood food residues (SPQ) had a slower viability loss rate than L. *rhamnosus* incorporated in the plain starch film (S), possibly due to the antioxidant effect of some components of the quince and potato peels. This result suggests that the use of agrifood residues in edible films may be a good strategy to slow down the viability loss rate of probiotics during storage. Microbiological studies revealed that, as expected, the agrifood residue powders contained substantial amounts of natural contaminant bacteria, which were almost completely eliminated in some formulations during the film forming process. Furthermore, none of the target foodborne pathogens -*Camphylobacter* spp., *Listeria monocytogenes*, or *Salmonella* spp. - were detected in any of the samples analyzed.

Overall, the results showed that these edible films may be used for food packaging and are promising carriers for probiotics, especially those composed of quince and potato peels.

CRediT authorship contribution statement

Patrícia Coimbra: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Susana Alarico:** Methodology, Investigation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Nuno Empadinhas:** Resources, Writing – review & editing. **Mara E.M. Braga:** Conceptualization, Methodology, Resources, Writing – review & editing, Funding acquisition, Project administration. **Marisa C. Gaspar:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by “Santander Universidades and University of Coimbra” through the project “Projetos Semente Santander UC: Era Pós-COVID-19: uma sociedade mais sustentável, resiliente e justa” and by Fundação para a Ciência e a Tecnologia (FCT): FCT/MCTEs (UIDB/00102/2020 and UIDP/00102/2020, of CIEPQPF, UIDB/04539/2020, UIDP/04539/2020 and LA/P/0058/2020, of CIBB, and UI/05704/2020 of ciTechCare. M. C. Gaspar acknowledges FCT for the financial support through the Institutional Scientific Employment Stimulus (CEECINST/00060/2021).

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