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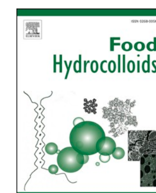

Physical properties and antimicrobial activity of bioactive film based on whey protein and *Lactobacillus curvatus* 54M16 producer of bacteriocins

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A B S T R A C T

The objective of the work was to study the viability and antimicrobial activity of bacteriocin-producing lactic acid bacteria (LAB) incorporated into whey protein/inulin/gelatine (WP) edible films in presence or absence of nutrient (modified MRS broth). Moreover, the role of the cell on the film structure and properties has been investigated. The results of the work showed that WP-based films were able to ensure a high viability of the bacteriocin-producing strain *L. curvatus* 54M16 during 28 days of storage at 4 °C. The addition of nutrient in the film matrix slightly affected the viability of the cells, but it was critical for the antimicrobial activity of the films. Films in presence of nutrient showed a good antimicrobial activity against *L. innocua* C6 as in vitro system as on cooked ham. The presence of LAB has a significant effect on the structure of the film: it reduced the viscosity of the film forming solution and improved the elasticity and the percentage of elongation. Whereas, no effect was observed for water vapour transmission rate and solubility. Thus, WP-based films in presence of modified MRS broth can be used as effective delivery and carrier systems for lactic acid bacteria to develop bioactive edible film or coating with antimicrobial properties.

1. Introduction

Packaging plays a critical role in the food supply chain. New packaging technologies designed to improve food safety and quality could help to reduce food loss during distribution chain. Active packaging deliberately incorporates active components intended to release or to absorb substances into, onto or from the packaged food or the environment surrounding the food in order to improve the quality of packaged food or to extend its shelf life (Regulation (EC) No 1935/2004). Among the many types of active packaging, in the last years antimicrobial active packaging is being increasingly experimented with the main goal of preserving the food during storage from microbial contamination and proliferation of pathogenic and spoilage microorganisms (Appendini & Hotchkiss, 2002; Bolívar-monsalve, Ramírez-toro, & Bolívar, 2019; Quintavalla & Vicini, 2002). The most studied antimicrobial food packaging systems have been classified according to their active compound: essential oils, enzymes and bacteriocins, organic acids and their derivatives (Yildirim et al., 2018). Among bacteriocin based active film, nisin-based active packaging is the most studied in food applications (Ercolini et al., 2010; Ferrocino et al., 2013; La Storia et al., 2012; Blanco Massani, Morando, Vignolo, & Eisenberg, 2012; Mauriello, De Luca, La Storia, Villani, & Ercolini, 2005) and it is still the only bacteriocin legally approved as food additive to be used by the food industry (Kumariya, Kumari, Rajput, Sood, & Akhtar, 2019). An alternative novel and natural preservation method is the incorporation of viable lactic acid bacteria (LAB) strains into a film or coating matrix for bacteriocin production during food storage (Concha-Meyer, Schöbitz, Brito, & Fuentes, 2011; Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010; Iseppi, Niederh, Anacarso, Messi, & Sabia, 2011; Leonard et al., 2015; Pereira, Soares, Costa, Silva, & Gomes, 2019; Pereira, Soares, Sousa, Madureira, Gomes, Pintado., 2016; Pereira, Soares, Monteiro, Gomes, & Pintado, 2018; Sánchez-gonzález, Iván, Saavedra, & Chiralt, 2013). Protective and probiotic bacterial cultures of LAB have traditionally used in the food production and are considered to meet the specific safety requirements that identify the microorganisms as Generally Recognized As Safe (GRAS, in the US) and with a Qualified Presumption of Safety (QPS, in the EU) (EFSA, 2016).

Edible coating and film can be obtained by using biopolymers,

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including proteins, polysaccharides or their combination (Bruno, Giancone, Torrieri, Masi, & Moresi, 2008; Giancone et al., 2011; Volpe, Cavella, Masi, & Torrieri, 2017). Among biopolymers, proteins have received considerable interest since it provides a film with distinct and valuable properties. Whey proteins are edible, biodegradable and have interesting mechanical properties. The film-forming properties of this type of protein are useful to produce transparent, flexible, colourless and odourless films (Calva-Estrada, Jiménez-Fernández, & Lugo-Cervantes, 2019). Gelatin, obtained from the partial hydrolysis of collagen, is a natural water-soluble protein characterized by the absence of an appreciable odour and by a linear structure (Coltelli et al., 2016). It has the capacity to form a soft, flexible, and elastic gel that confer a less organized matrix when blended with other protein, such as whey protein (Calva-Estrada et al., 2019). Pereira et al. (2019) showed that whey protein films were more effective in preserving *Bifidobacterium animalis* subsp. *lactis* BB-12 viability compared to alginate films and the most effective results were obtained with inulin incorporation. Inulin is a dietary fiber, known for its properties as prebiotic and functional ingredient (Shoaib et al., 2016). Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, and Fisk (2014) showed that the cell viability of probiotic strain was higher in film containing inulin for its prebiotic function. Garcia-Argueta et al. (2013) showed that edible film based on whey protein, gelatin and inulin assured the survival of *Lactobacillus casei* for 10 days at 25 °C. The LAB cells viability and the antimicrobial activity can be affected by the structure and composition of the biopolymer film (Léonard et al., 2013; Leonard et al., 2015). Moreover, it is also important to know the effects of the incorporated active compound on the physical and structural properties of the films. It can lead to either a positive or a negative effect depending on both the nature of the active compound and of the biopolymers used as matrices (Benbettaïeb, Karbowiak, & Debeaufort, 2019b; Lago et al., 2014; Perone, Torrieri, Cavella, & Masi, 2014; Torrieri, Cavella, & Masi, 2015). Thus, the objectives of the present study were to study (i) the viability of *Lactobacillus curvatus* 54M16 cells dispersed into a whey protein/gelatin/inulin film in presence or absence of a modified MRS broth; (ii) the impact of the bacterial cells on physical and structural properties of the films and (iii) the antimicrobial effectiveness of the films against *L. innocua* C6 growth in laboratory media and an actual food model system.

2. Materials and methods

2.1. Materials

Whey milk proteins isolate (WPI) with a high-purity grade (92%) were provided by A.C.E.F. s.p.a. (Piacenza. Italy). Glycerol was purchased from Merck KGaA EMD Millipore Corporation (Darmstadt, Germany). Gelatin (100% purity; bovine origin; Cameo s.p.a., Desenzano del Garda, Italy) was purchased at the Euroesse supermarket (Portici. Italy). Inulin (Farmalabor Srl., Canosa di Puglia, Italy) was purchased at the ALMA pharmacy (Portici. Italy). Sodium Acetate and Tris-HCl were purchased by Sigma Chemical Co. (St. Louis. MO. USA). All the culture media were purchased from Oxoid (Rodano, Milano, Italy).

2.2. Bacterial strains and growth conditions

Lactobacillus curvatus 54M16 was isolated from traditional fermented sausages and produces the bacteriocins *sak* X, *sak* T_α, *sak* T_β and *sak* P (Casaburi, Di Martino, Ferranti, Picariello, & Villani, 2016; Giello, La Storia, De Filippis, Ercolini, & Villani, 2018). The strain was stored at −20 °C in MRS broth (Oxoid) supplemented with 25% (v/v) sterile glycerol. Before use, the strain was sub-cultured twice in a modified MRS broth (m-MRS) at 30 °C. The composition of the m-MRS broth was (g/L of distilled water): 8.0 Lab-Lemco, 10 Peptone, 4.0 Yeast Extract, 20 Dextrose, Tween 80 (1 ml/L). A 10 ml aliquot of reactivated culture was transferred into 1000 ml of MRS broth and incubated for 24 h at 30 °C. The culture was centrifuged at 6500 *g* for 20 min at 4 °C and the cell pellet was washed twice with phosphate buffer (PBS, Oxoid) and suspended in 15 ml of sterile distilled water or m-MRS broth (about 10 log CFU/ml).

Listeria innocua C6 (Casaburi et al., 2016), used as bacteriocin indicator, was grown in Trypticase Soy Broth supplemented with 0.5% yeast extract (TSBY, Oxoid) at 30 °C.

2.3. Film preparation

The formulation of the film forming solutions (FFS) was reported in Table 1. Gelatin and inuline were included in the formulation due to their ability to form gels and to their physicochemical properties that create synergy among the components to enable the formation of three-dimensional networks. Moreover, inuline has also a prebiotic function. Three different films were prepared: a) control film prepared without *Lb. curvatus* 54M16 (WPC); b) bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth (WPM), c) bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water (WPH). Culture of *Lb. curvatus* 54M16 was added to film forming solutions at 4% (v/v) to attain a final concentration of 8.56 and 8.54 Log CFU/mL for WPM and WPH film, respectively.

Glygerol, whey protein, inulin, gelatin and m-MRS broth were dissolved in deionised water under continuous stirring at 50 °C for 30 min (WPC). Then, the solution was cooled down to 30 °C and enriched with *Lb. curvatus* 54M16 at 4% (v/v) under continuous stirring for 15 min (WPM). WPH samples were obtained as the same WPM sample, but the m-MRS broth was replaced by deionised water. The pH of WPC was 5.85, whereas the pH of WPH and WPM was 5.95 and 6.01, respectively. Films were obtained by casting: five ml of each FFS were poured into Petri dishes (surface of 56.7 cm^2) and allowed to dry at 30 °C and 50% relative humidity (RH) for 24 h in circulating air system chamber (MMM Medcenter Einrichtungen GmbH, Monaco di Baviera, Germany). Dried films were peeled from the Petri dishes and stored at 4 °C and 50% RH prior to testing.

2.4. Rheological properties of FFS

The FFS viscosity was measured as reported in Volpe et al. (2017) with small modifications. A strain-controlled rheometer (HAAKE MARS 40 Rheometer, Thermo Fisher Scientific, USA) equipped with coaxial cylinders (30 mm o.d. and 26 mm i.d.) has been used. Measurements were performed at 30 °C by increasing the shear rate from 0.1 to 100 s^{-1}. All measurements were replicated three times. The steady shear viscosity has been calculated with respect to the following equation:

$$\tau = \eta \gamma \quad (1)$$

where τ is the shear stress (Pa), γ is the shear rate (s^{-1}) and η is the viscosity (Pa s).

Table 1
Film forming composition of bioactive WP-based film.

Composition	Film samples[a]		
	WPC	WPM	WPH
Whey Protein isolate (%w/v)	13.0	13.0	13.0
Edible gelatin (%w/v)	6.0	6.0	6.0
Inulin (%w/v)	4.0	4.0	4.0
Glycerol (%w/v)	6.0	6.0	6.0
m-MRS broth (%v/v)	15.0	15.0	0.0
Cells of *Lb. curvatus* 54M16 (%v/v)	0.0	4.0	4.0
Deionised water (%v/v)	56.0	52.0	67.0

[a] : WPC, control film prepared without *Lb. curvatus* 54M16; WPM, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth; WPH, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water.

2.5. Viability of L. curvatus 54M16 during storage of the film

Viable counts of *L. curvatus* 54M16 were determined in the FFSs just before the casting process, while the viability of the microorganisms was determined in the films after 1, 2, 7, 15 and 28 days of storage at 4 °C.

FFS was diluted 1:10 in quarter-strength *Ringers solution* (Oxoid) and aliquots of serial decimal dilutions were poured in duplicate plates of MRS agar. The plates were incubated in anaerobiosis (AnaeroGen, Oxoid) at 30 °C for 48 h. The results were expressed as log CFU/ml.

The films (WPC, WPM and WPH) at each sampling time were placed in a Stomacher bag with 10 ml of *Ringers solution* (Oxoid). The bag containing each film was immersed in a water bath at 40 °C for 30 s for the complete dissolution and release of the cells trapped in the film and then homogenized by stomacher for 5 min. Sequential decimal dilutions were made in Ringer's solution. Each dilution was plated on MRS agar and incubated in anaerobiosis at 30 °C for 48 h. The results were expressed as means of log CFU/cm^2.

2.6. Film characterization

2.6.1. Scanning electron microscopy

The film surface and the fracture surfaces (transversal area) obtained after immersing film samples in liquid nitrogen (fragile fracture) were observed using a scanning electron microscope (LEO EVO 40, Zeiss, Oberkochen, Germany). The films were mounted on bronze stubs using double-sided tape and then placed on specimen stubs with the cross-section oriented upward and were coated with a thin layer of gold using a DC sputter coater (AGAR B7340, Agar Scientific Ltd, Stansted, UK). Digital images were collected at a tilt angle of 0° to the electron beam using an acceleration voltage of 20 kV. SEM analysis were performed on samples WPC and WPM.

2.6.2. Physiochemical properties

The film surface density (ρ) was calculated as:

$$\rho = \frac{C \cdot V}{A} \tag{2}$$

where C is the blend solution concentration (mg ml^{-1}), V is the volume of the solution poured into the petri dish (ml) and A is the surface area of the petri dish (cm^2).

Film thickness was measured using a micrometer model H062 with sensitivity of ±2 μm (Metrocontrol Srl, Casoria, NA, Italy). Five replications were conducted for each sample treatment. Five measurement were taken at random position around the film sample.

The moisture content of the films was evaluated by gravimetric method, maintaining the film at 105 °C for a time enough to reach a constant weight. The results are expressed as relative humidity percentage (UR%) calculated as:

$$UR\% = \frac{(p_i - p_f)}{p_i} \cdot 100 \tag{3}$$

"p" is the weight of the film (g), "i" and "f" correspond to the initial and final weight of the film. For each sample, three replicates were performed.

The film opacity was determined according to the method described by Siripatrawan and Harte (2010), by measuring the absorbance at 600 nm using the UV-VIS spectrophotometer (Jasco V-550 UV/VIS Spectrophotometer). The films were cut into rectangular pieces 3 cm high and 0.4 cm wide and placed directly into the cuvette of the spectrophotometer. The opacity of the film was calculated with the following equation:

$$T = \frac{Abs600}{x} \tag{4}$$

"T" is transparency, "Abs600" is the absorbance value at 600 nm and "x" is the thickness of the film (mm). For each sample five replicates were performed.

The colour of the samples was evaluated using a colorimeter (Minolta CHROMA METER CR-300, Japan). The colour values of L (black/white), a*(redness/greenness) and b* (yellowness/blueness) were measured and averaged from five random positions for each sample, and the total colour difference (ΔE) was calculated according to Pereira et al. (2016).

The solubility of the films at different pH was tested with the procedure reported by (Giancone et al., 2011). Small pieces of film (20 mg) were dried in an oven at 105 °C for 24 h and weighed with a value closer to 0.0001 g to determine the initial dry weight of the film. Each piece of film was incubated at 25 °C for 24 h in a falcon containing 10 ml of an acetate solution [0.1 M] (pH 4), distilled water (pH 6), and Tris-HCl (pH 8). At the end of the incubation, the samples were recovered on a Whatman no. Filter. 1. The part of the undissolved film was removed from the filter using 10 ml of distilled water and dried in a vacuum oven at 70 °C and 6.67 kPa for 24 h and finally weighed. The solubility of the film (FS%) was calculated as follows:

$$FS\% = \frac{w_i / w_f}{w_i} \cdot 100 \tag{5}$$

"w" is the dry substance, "i" and "f" correspond to the initial and final dry matter.

For each sample, three replicates were performed.

2.6.3. Mechanical analysis

Dynamic mechanical analyses were performed using a DMTA V (Rheometrics, Inc., Piscataway, USA) that applies an oscillatory force at a set frequency to the sample and reports changes in stiffness and damping on rectangular film strips (20 ×7 mm). Each sample was cut with scissors and mounted on grips so that its effective length was 10 mm. All the samples were submitted to a strain sweep test at a given frequency (ω) of 1 rad s^{-1} to determine the linear viscoelastic region. Then, a frequency sweep test was carried out applying a strain amplitude (ε) of 0.005% (within the linear viscoelastic region) and increasing the frequency from 10^{-2}–10^{3} rad s^{-1} to monitor the storage modulus (E′) that measure of the sample's elastic behaviour, and the tangent delta (tanδ), that is the ratio of the loss storage and the storage modulus. All measurements were conducted in the dynamic mode and the results were reported as average of three replicates.

Tensile tests were carried out by using an Instron Universal Model No 4301 (Instron Engineering Corp., Canton, MA) at room temperature, according to a standard test method (Method D882, ASTM, 2001). The instrument was equipped with a 1 kN load cell and the crosshead speed was equal to 50 mm min^{-1}. Films were cut into 25-mm wide and 100-mm long strips. Elastic modulus (E), tensile strength (TS) and elongation at break (ε%) were calculated. The results were reported as average of seven replications of each sample.

2.6.4. Water vapour permeability

The water vapour permeability (WVP) of the films was evaluated as reported by Volpe et al. (2017) using a gravimetric test according to ASTM E 96 (1993) by means of Payne permeability cup (Carlo Erba, Milan, Italy). The Water vapour permeability (WVP) was calculated at 20 °C and at 85% of RH, as:

$$\mathrm{WVTR} = \frac{dm}{\mathrm{dt}} \cdot \frac{x}{\mathrm{A} \cdot \Delta \mathrm{p}} \tag{6}$$

where $\frac{dm}{dt}$ is the slope of the weight curve with respect to time after reaching the steady state, "x" represents the thickness, "A" is the exposed area of the film (9.89 cm^2), "Δp" is the vapour pressure water through the film. The results are reported as the average of three replications of each sample.

2.6.5. Antimicrobial activity

Antimicrobial activity of the films during storage at 4 °C was detected against the strain of *L. innocua* C6 as previously described by Mauriello et al. (2005), with some modifications. At each sampling time, film pieces (ca 2×2 cm^2) were aseptically cut from each film and placed on TSA plates. After incubation at 4 °C for 3 h to allow bacteriocins diffusion, the plates were covered with TSA soft agar (0.75% agar) inoculated with about 10^6 CFU/ml of an overnight culture of the indicator strain. Plates were incubated at 30 °C, 10 °C and 4 °C for 24 and 72 h and 20 days, respectively. After incubation the inhibition zones of the indicator organism around the films were determined and expressed in cm^2. Each value was the mean of two experiments with three replicates each.

The antagonistic activity of the active films was also evaluated by determining *L. innocua* counts, as described by Sánchez-González, Quintero Saavedra, and Chiralt (2014), with some modifications. Overnight culture of *L. innocua* C6 was inoculated (about 10^5 CFU/cm^2) on the surface of solidified TSA plates, which were then covered with active (WPM) and non-active (WPC) films with the same size as the Petri dishes. The counts of *L. curvatus* 54M16 and *L. innocua* C6 were determined after 0, 1, 7, 15 and 28 days of storage at 4 °C. At each sampling time the agar covered with the films was withdrawn aseptically from the Petri dishes and transferred in a Stomacher bag with 100 ml of quarter strength Ringer's solution (Oxoid). The content of the bag was first manually ground and then the bag was immersed in a water bath at 40 °C for 30 s for the complete dissolution and release of the cells trapped in the film, and finally homogenized by stomacher for 5 min. Sequential decimal dilutions were made in Ringer's solution. Each dilution was plated on MRS agar incubated in anaerobiosis (AnaeroGen, Oxoid) at 30 °C for 48 h and on ALOA (Biolife, Milano, Italy) incubated at 30 °C for 48 h. The results were expressed as means of log CFU/cm^2.

2.7. Antimicrobial effect of the films during the storage of cooked ham

Cooked ham discs (56.7 cm^2 × 0.5 cm thick) were aseptically cut and transferred into sterile Petri dishes. Overnight culture of *L. innocua* C6 was spread on the surface of ham to obtain a final concentration of approximatively 10^5 CFU/cm^2. The ham discs were covered with the WPC and WPM films. After 0, 1, 7, 15 and 28 days of storage at 4 °C, the samples were homogenized with 100 ml of quarter strength Ringer's solution (Oxoid) for 2 min by Stomacher at room temperature. Decimal dilutions of the homogenates were prepared in the same diluent and selective viable counts of Listeria on ALOA (Biolife) were performed. The plates were incubated at 37 °C for 48h and the results were expressed as means of log CFU/cm^2.

LAB viability in WPM film covering the cooked ham was also determined during each time as described above.

2.8. Data analysis

All experimental results are reported as mean value ± standard deviation. Data were analysed using variance to study the effect of film composition (WPC, WPM, WPH) on film functional properties. Only for the cell viability and antimicrobial properties of the film, the effect of storage time was also investigated. Five level of storage time were studied (0, 1, 7, 15, 28 days). Duncan's test was carried out to find the source of the significant differences within the samples examined. Significant differences were defined at $p < 0.05$. All statistical analyses were performed using the SPSS software (SPSS Inc. 17.0, Chicago, 2002).

3. Results and discussions

3.1. Rheological properties of film forming solution

All solutions had a Newtonian behavior. ANOVA showed that the presence of LAB had a significant effect on viscosity ($p < 0.05$), which was equal to 0.11 ± 0.01 (Pa s) for WPC samples and assumed an average value of 0.07 ± 0.01 (Pa s) for WPM or WPH samples. Thus, WPC sample showed higher viscosity values than WPH and WPM samples. Kanmani and Lim (2013) showed that the addition of probiotic bacterial strains in edible film based on pullulan and starch decreased the viscosity and pH of all film-forming solutions. García-argueta, Dublán-garcía, and Quintero-salazar (2013) studied the effect of inulin, gelatin and LAB on viscosity of WP based film and showed that gelatin and gelatin–LAB interaction decreased the viscosity of the film forming solution. The presence of LAB cell can create discontinuity in the film forming solution that induces a different interaction among whey protein, gelatin and inulin. Since molecular mass affects the dependence of viscosity on the shear rate (Williams & Phillips, 2000), the lower viscosity can be explained by an increment of free volume due to the presence of bacteria cells included in the protein complex.

3.2. Viability of L. curvatus 54M16 during storage of the film

Ensuring the viability and functionality of microorganisms incorporated in a bio-polymer matrix represent the main conditions to guarantee the effectiveness of active films.

The viability of the producing bacteriocins strain of *Lb. curvatus* 54M16 included in WPM film (prepared by adding nutrients resulting from a modification of the MRS broth (m-MRS)) and in WPH film (prepared without m-MRS) was determined during the storage of the films at 4 °C for 28 days. Viable counts on MRS agar plates are shown in Table 2. The *Lb. curvatus* count in WPM film remained stable at level ranging from 7.67 to 7.58 log CFU/cm^2 during the storage period. In WPH film a significant reduction, though with a very slight decrease (0.47 units after 28 days of storage at 4 °C) of the initial population of the strain was observed at 15 days and 28 days of storage, indicating that the presence of nutrients (m-MRS) in the composition of the film influences the viability of the microorganism.

Literature reviews show that similar films based on whey protein, gelatin, inulin and glycerol guarantee the survival of different strains of lactic bacteria during storage and their use as a coating for different foods (Garcia-Argueta et al., 2016, 2013; Pereira et al., 2019; Shoaib et al., 2016). Moreover, protein-based films appear to ensure greater viability of the microorganisms added to the matrix compared to films based on cellulose derivatives (Sánchez-gonzález et al., 2013; 2014). The results of this study showed that the optimal formulation of edible films to ensure a high viability of the bacteriocin-producing strain *L. curvatus* 54M16 was 13% whey protein, 4% inulin, 6% gelatin, 6.0% glycerol, 4% of bacterial cells and 15% of m-MRS broth (Table 1). Nevertheless, both films, with and without nutrients, can be considered as a good carrier of viable cells, even if the presence of nutrients is critical for the antimicrobial activity of the films, as discussed in

Table 2
Viability of *L. curvatus* 54M16 in WP-based edible films during storage at 4 °C.

Films[a]	Viable cell numbers (Log CFU/cm^2) at days[c]				
	1[b]	2	7	15	28
WPC	**< 1**	**< 1**	**< 1**	**< 1**	**< 1**
WPM	**7.67 ± 0.01^{aA}**	**7.69 ± 0.09^{aA}**	**7.66 ± 0.04^{aA}**	**7.51 ± 0.03^{aA}**	**7.58 ± 0.01^{aA}**
WPH	**7.59 ± 0.08^{aA}**	**7.49 ± 0.17^{aA}**	**7.51 ± 0.01^{bA}**	**7.41 ± 0.02^{bB}**	**7.36 ± 0.01^{bBC}**

[a] WPC, control film prepared without *Lb. curvatus* 54M16; WPM, film prepared adding *Lb. curvatus* 54M16 and mMRS broth; WPH, film prepared adding *Lb. curvatus* 54M16 and distilled water.

[b] Films immediately after castings at 30 °C for 24 h.

[c] The values are the means ± SD obtained from four plates of three independent trials. Means within a row with the same capital letter are not significantly different ($P > 0.01$); Means within a column with the same lower case letter are not significantly different ($P > 0.01$).

paragraph 3.3.4.

3.3. Film characterization

3.3.1. Scanning electron microscopy analysis

Fig. 1 shows the microstructure of WPC and WPM films. No differences were observed between the cross section of WPC and WPM, since both films were characterized by a compact, homogeneous and uniform structure. Similar results were reported by Pereira et al. (2016) who showed how the incorporation of probiotic strains in the edible film did not confer any noticeable modification to the structural conformation of the films. However, it was not possible to distinguish the presence of bacteria cells for WPM films. It is probable that the bacteria cells were well incorporated into the matrix, so that resulted in a non-discontinuous structure. By looking at the surfaces of the films, it is possible to notice that the surface of WPC resulted rough and uneven due to the formation of steam bubbles during the film casting. On the other hand, the surface of WPM was smooth and compact. The different surface of the films can be explained by the different rheological properties of the FFS. Indeed, the higher viscosity of WPC did not allow to the steam bubble to evaporate, so that the bubbles remain in the matrix.

3.3.2. Physiochemical properties of the film

Film surface density, thickness, moisture content, colour, and opacity of the films are reported in Table 3. The surface density of both WPC and WPM films was 0.17 g cm^{-2}, while for the WPH film it was 0.15 g cm^{-2}. The thickness of WP-based film ranged from 130 $\pm$ 23 μm for WPH film to 162 $\pm$ 30 μm for control film (WPC). The lower thickness value of WPH film respect to WPC and WPM film can be related to its lower solid content. However, based on statistical analysis, no significant difference among samples were highlighted ($p > 0.05$). Thus, the presence of the m-MRS broth at 15% or the presence of the LAB cell did not affect the film thickness.

Moisture content of film was not affected by film formulation ($p >$ 0,05) (Table 3). It assumes an average value of 14 $\pm$ 1%. Optical properties (opacity and colour) had a direct impact on the visual appearance of the film. All films showed high brightness values, demonstrating that films appeared clear and transparent. The lightness value (L*) of WPC film showed a higher value respect to WPM and WPH film (Table 3). Statistical analysis highlighted a significant difference among samples. Moreover, from the results of Duncun test, it can be observed that samples WPM and WPH were no statistically different. Thus, the different lightness of the film is due to the presence of the cells. Same results were reported also for the parameters a*. WPC sample showed a higher a* value compared to WPM and WPH film. For b* parameter, a statistical difference between WPH and WPM was observed too ($p < 0.05$). In presence of cell and with m-MRS broth the highest b* value is obtained. The total colour difference (ΔE) showed a significant difference of WPC sample from WPM and WPH, as for L* and a* ($p <$ 0,05). Same results were obtained for the opacity of the film (Table 3). Therefore, the addition of lactic acid bacteria cells to the film-forming solution affects the appearance of the films.

Similar results were reported by Bekhit et al. (2018), who showed that the disperse bacterial cells, with a different refractive index, decreased the transparency of the film due to the enhancement of the light scattering, the films lightness and whiteness index.

Solubility is an important parameter that measures the water resistance and the integrity of film; therefore, the film solubility at different pH has been calculated. The film solubility ranged between 25% and 30% as function of film composition and pH (data not shown). ANOVA showed that there was no significant difference among samples at single pH values ($p > 0.05$) and that the highest solubility values (about 30%) was obtained at pH 6. Therefore, these films in contact with acidic foods (pH 4) or basic foods (pH 8) did not solubilise as much as when in contact with foods that have a pH tending to neutrality. The results can be justified by the relation between solubility and isoeletric point of the proteins. The pH of the solution affects the nature and the distribution of the protein's net charge. Generally, the proteins are more soluble at low (acids) or high (alkaline) pH values because of the excess of charges allowing the water to interact with protein (Pelegrine & Gasparetto, 2005). In any case, the solubility value was not very high. This indicates the high cohesion of the matrix.

3.3.3. Mechanical properties and water vapour permeability

Fig. 2 shows the dependence of the storage modulus (E′) and loss tangent (tan δ) on ω for the WPC and WPM films. E' value could be

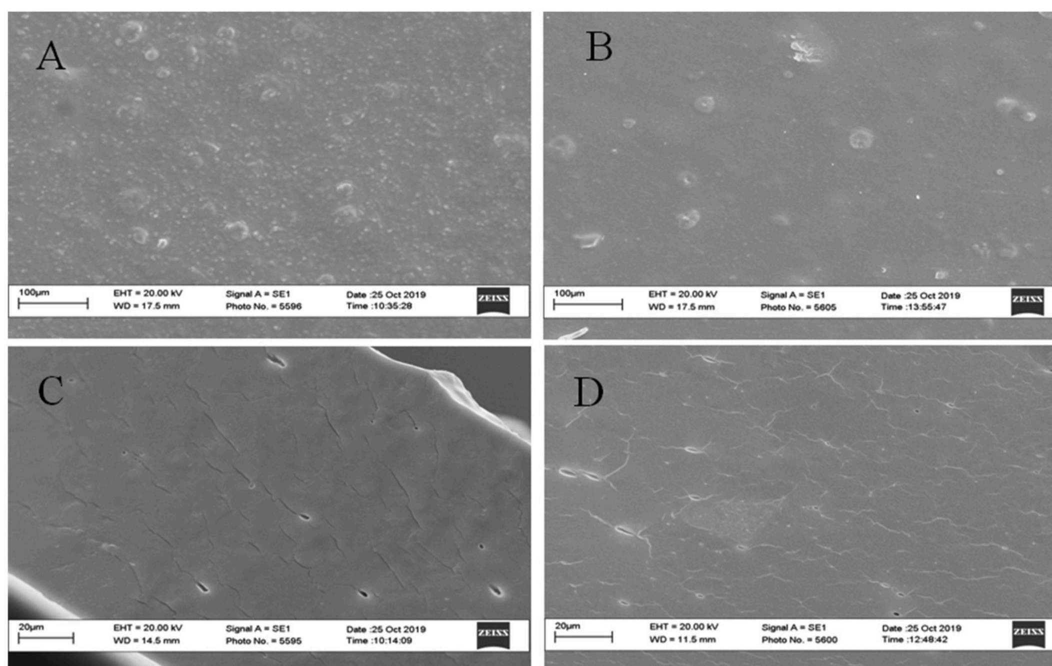

Fig. 1. SEM micrographs of the WP-based films: A) WPC film surface, B) WPM film surface, C) WPC film cross section; D) WPM film cross section. Magnification 500 x for the surface, 2000x for the cross section.

Table 3
Solid surface density (ρ), thickness (Δx), moisture content (MC), colorimetric parameters (L*, a*, b*, ΔE) and opacity of WP-based films.

Film	ρ_s (gcm^{-2})	Thickness (μm)	MC%	L	a*	b*	ΔE	Opacity
WPC	0.17	162 ± 30a	15±1a	96,9 ± 0,8^a	0,3 ± 0,2^a	1,3 ± 0,7^a	1±1^a	0,9 ± 0,1^a
WPH	0.15	130 ± 23a	14,8 ± 0,4a	93,6 ± 0,7^b	−0,6 ± 0,2^b	5,9 ± 0,6^b	4±3^b	1,16 ± 0,09^b
WPM	0.17	160 ± 25a	13±2a	93±1^b	−0,9 ± 0,1^b	7,5 ± 0,8^c	6±4^b	1,21 ± 0,09^b

WPC, control film prepared without *Lb. curvatus* 54M16; WPM, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth; WPH, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water. Means within a column with different letters are significantly different ($p < 0.05$).

related to how elastic the material is. The loss tangent is an indicator of how efficiently the material loses energy to molecular rearrangements and internal friction. It is calculated as ratio of the loss and the storage modulus and therefore is independent of geometry effects (Menard, 1999).

By increasing ω from 10^{-2}–10^2 rad s^{-1}, E′ increased for both the films; in particular, WPM showed the greatest E' value, which ranged from 3.49 × 10^9 Pa to 2.63 × 10^{10} Pa, whereas WPC started from 7.95 × 10^8 Pa until reaching 6.84 × 10^9 Pa. Tan δ of WPC decreased from 1.55 to 0.829, whereas WPM had lower values, ranged from 0.839 to 0.343. The results showed that the incorporation of bacterial cells into the matrix influenced the structure of films; WPM film showed more elasticity compared to the WPC film.

The tensile properties of the film are reported in Table 4. WPC film showed a significantly higher elastic modulus values (E > 40%), higher tensile stress values (TS > 30%) and lower percentage of elongation (ε<29%) respect to the films inoculated with strain 56M16, either in presence or in absence of m-MRS broth (WPH and WPM) ($P < 0.05$), but no significant differences were observed between films inoculated with strain (WPM and WPH) ($P > 0.05$). The elongation at break of WPC film was of the same order of magnitude as that of whey protein-based film (Fairley, Monahan, Bruce German, & Krochta, 1996; Galietta, Di Gioia, Guilbert, & Cuq, 1998).

The water vapour transmission rate (WVTR) and the water vapour permeability (WVP) values of whey protein-based films are reported in Table 4. The WVTR and WVP of WPC film were 55 ± 6 gm^{-2} s^{-1} and 39 ± 6 gm^{-2} s^{-1}Pa^{-1}, respectively. The WVTR and WVP were in the range of other bioactive edible film (Gialamas et al., 2010; Soukoulis et al., 2014). The presence of the cell or m-MRS broth didn't modify significantly WVP value. Similar results were reported by Bekhit at al. (2018) and Gialamas et al. (2010), who reported that the addition of the bioactive culture did not alter significantly the barrier properties of the films.

How the incorporation of the cell can affect the structure and functional properties of the film is still unclear. More is known on the incorporation of specific active compound. As well reviewed by Benbettaïeb, Karbowiak, & Debeaufort (2019), organic acid and antimicrobial protein or bacteriocins can have an effect positive or negative on the film structure and in turn of its mechanical and barrier properties as function of the possible interaction between the biopolymer and the antimicrobial agent. As general trend, if new strong interactions, such us covalent bond, are established in the biopolymer matrix, an anti-plasticizer effect is obtained with a positive impact on barrier properties. In fact, a way to improve the barrier properties of biopolymer film is to improve the cross-link in the structure. On the other hand, when the antimicrobial protein or bacteriocin disrupts the continuous network of the biopolymer matrix, a plasticizer effect is expected.

Table 4
Tensile and water vapour barrier properties of WP-based films.

Film	Tensile properties			WVTR × 10^4	WVP × 10^{11}
	E (MPa)	TS (MPa)	ε%	gm^{-2} s^{-1}	gm^{-1} s^{-1}Pa^{-1}
WPC	289 ± 96^a	6±2^a	16 ± 14^b	55±6^a	39±6^a
WPH	172 ± 51^b	4±1^b	54 ± 19^a	51±4^a	39 ± 15^a
WPM	151 ± 49^b	3,4 ± 0.7^b	44 ± 20a	52±3^a	28±5^a

EM: elastic modulus; TS: tensile strength; ε%: percentage of elongation; WVTR: water vapour transmission rate; WVP: water vapour permeability; WPC, control film prepared without *Lb. curvatus* 54M16; WPM, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth; WPH, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water. Means within a column with different letters are significantly different ($p < 0.05$).

Acidulant or bacteriocin might exert a plasticizing effect because they are small molecules with hydroxyl groups that replace protein–-protein interactions by developing protein-plasticizer hydrogen bonds, and so increase the water-content equilibrium, water vapour permeability, and extensibility that affects the glass-transition temperature of the film (Calva-Estrada et al., 2019).

Lactic acid bacteria added in the biopolymer matrix produce in situ bacteriocin that can interact with the film structure. The effect depends by the nature and concentration of compound produced, and by the nature of biopolymer. Bekhit et al. (2018) reported that the incorporation of lactic acid bacteria in both HPMC and corn starch films, didn't

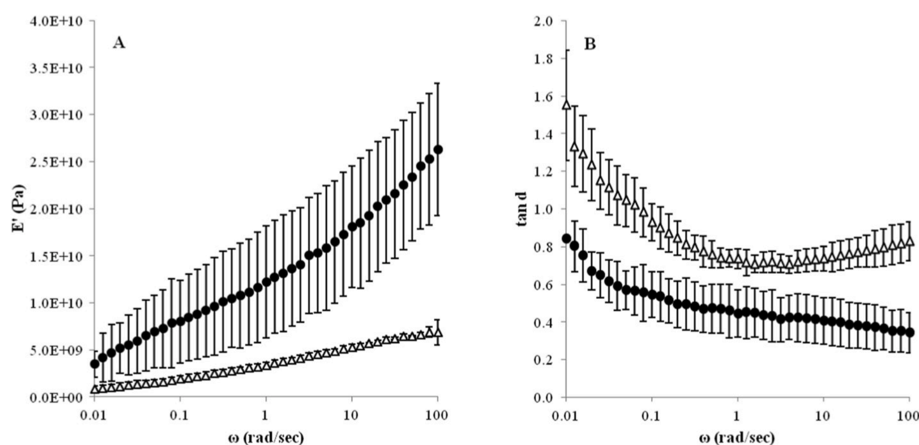

Fig. 2. Elastic modulus (E′) (A) and loss tangent (tan δ) (B) versus the angular frequency (ω) of the WP-based films: WPC (Δ), control film; WPM (●), film with inoculation of *Lb. curvatus* 54M16 and m-MRS broth.

modify significantly its barrier and mechanical properties. Whereas, for corn starch film, when L. lactis was incorporated in the matrix, an increase of percentage of elongation at break and a notable decrease of elastic modulus was observed. Moreover, García-argueta et al. (2013) studied the effects of different components (whey protein, glycerol, inulin and gelatin) and the addition of probiotic bacteria of lactic acid bacteria (LAB, *Lactobacillus casei*) on edible films; they found that an interaction between LAB and inulin occurred, leading to an increase in film elasticity.

3.3.4. Antimicrobial activity of the films throughout storage

The strain of *L. innocua* C6 was used as indicator organism in all the experiments to detect the antimicrobial activity of the films. The strain showed a similar growth rate but a lower sensitivity to the bacteriocins produced by *L. curvatus* 54M16 when compared with *L. monocytogenes* ATCC 7644 (Casaburi et al., 2016). Antimicrobial activity of the films during storage at 4 °C for 28 days was determined on plates of *L. innocua* C6 incubated at 30 °C, 10 °C and 4 °C.

The results showed that the presence of nutrients and the temperature at which antagonism was detected were critical for the antimicrobial activity of the films. As shown in Table 5, no significant decrease in activity was detected during the storage period for WPM films at 30 °C, 10 °C and 4 °C and for WPH films at 4 °C; instead, a significant gradually decrease of activity was observed in WPH film during the 28-day period at both 30 °C and 10 °C (Table 5), attributable to a lower production of bacteriocins by *L. curvatus* 54M16 in the absence of nutrients. Moreover, control films (WPC) prepared without *Lb. curvatus* 54M16 did not show antimicrobial activity against the indicator microorganism. A gradual reduction of antimicrobial activity of starch, alginate and D-MRS broth-based films containing cells of two bacteriocin-producing lactic acid bacteria was also observed by Concha-Meyer at al. (2011). The decrease of activity was attributed to a possible death of the bacteria due to a reduction of the nutrients during the storage of the film. These and many other findings from literature reviews confirm that choice of the microorganism and nature of the film matrix represent determining conditions for bacterial vitality and antimicrobial action of the films (Gialamas et al., 2010; Sánchez-gonzález et al., 2013; 2014).

Moreover, in this study, for both WPM and WPH films, the inhibition areas were significantly wider when the antagonism was detected at 4 °C and 10 °C (Table 5, Fig. 3). This may be due to a better diffusion of the bacteriocins from the film and/or to different growth dynamics of the indicator strain in the presence of bacteriocins at temperatures of 4 °C, 10 °C and 30 °C.

Table 5
Antimicrobial activity of WP-based films containing *L. curvatus* 54M16 against *L. innocua* C6 during storage of the films at different temperature.

Time (day)	Inhibition zones (cm^2)[a]					
	WPM[b]			WPH[c]		
	30[d]	10[e]	4[f]	30	10	4
1	9.0 ± 0.2^{Aa}	12.9 ± 0.2^{Ba}	13.7 ± 0.2^{Ca}	5.3 ± 0.1^{Da}	7.84 ± 0.08^{Ea}	7.41 ± 0.06^{Ea}
7	8.9 ± 0.5^{Aa}	12.1 ± 0.3^{Ba}	13.5 ± 0.2^{Ca}	4.9 ± 0.4^{Db}	7.7 ± 0.1^{Ea}	7.36 ± 0.05^{Ea}
15	9.0 ± 0.1^{Aa}	12.9 ± 0.1^{Ba}	13.56 ± 0.05^{Ca}	4.84 ± 0.05^{Db}	6.7 ± 0.1^{Ec}	7.65 ± 0.04^{Ea}
28	9.1 ± 0.3^{Aa}	12.4 ± 0.4^{Ba}	13.5 ± 0.1^{Ca}	4.6 ± 0.3^{Dc}	6.6 ± 0.2^{Ec}	7.6 ± 0.1^{Ea}

a: areas of inhibition including the area of the film; b: WPM, film prepared adding *Lb. curvatus* 54M16 and m-MRS broth; c: WPH, film prepared adding *Lb. curvatus* 54M16 and distilled water; d, e and f: plates with indicator strain were incubated at 30 °C for 24 h, at 10 °C for 72 h and at 4 °C for 20 days, respectively. Control film (WPC) prepared without *Lb. curvatus* 54M16 did not show antimicrobial activity against the indicator microorganism. Means within a row with the same capital letter are not significantly different (P > 0.01); means within a column with the same lower-case letter are not significantly different (P > 0.01).

The antimicrobial activity was also evaluated against *L. innocua* C6 inoculated (about 10^5 CFU/cm^2) on the surface of TSA plates that were then covered with WPM and WPC films and stored at 4 °C for 28 days.

The results of *L. innocua* counts, reported in Table 6, showed that the WPM film determined a significant decrease of the microorganisms compared to the control film (WPC). The decrease of *L. innocua* was about 2 log cycles after only 24 h, reaching values below the detection limits at the end of the storage period. The results confirmed the bactericidal action of the bacteriocin produced by *L. curvatus* 54M16 (Casaburi et al., 2016). On the contrary, the film without *L. curvatus* 54M16 (WPC) did not cause inhibition of *L. innocua* that increased from 5.25 to 6.42 log CFU/cm^2 after 28 days of storage. In a similar in vitro antimicrobial test against a strain of *L. innocua*, Sanchez-Gonzales et al. (2014) reported a reduction of the microorganism of 1.5 log cycles for methylcellulose and sodium-caseinate-based films containing cells of bacteriocin-producing *L. acidophilus* and *L. reuteri* compared to the control films, whereas, a 3 log reduction of *L. monocytogenes* was reported by Gialamas et al., 2010, for sodium-caseinate films containing cells of bacteriocin-producing *L. sakei*.

In our study, *L. curvatus* count remained somewhat stable after the contact of the WPM film with the surface of the medium during the 28 days of storage at 4 °C (Table 6). However, the bacteriocin-producing strain assured the protective effect of the film throughout the storage period.

Finally, the antimicrobial activity of the WPM film was tested in a food model system consisting of cooked ham. Even in this experiment,

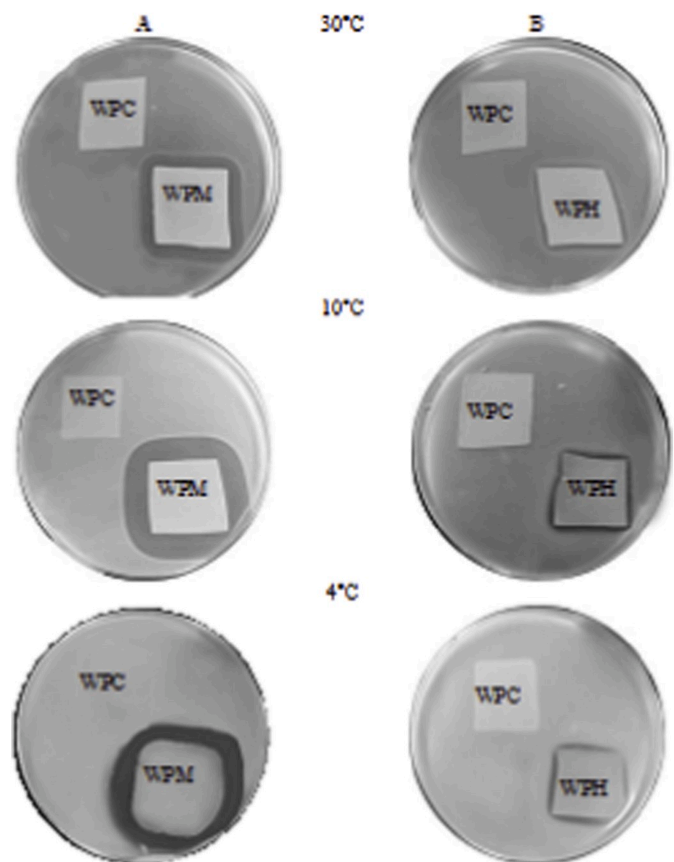

Fig. 3. Examples of inhibition zones of WP-based films containing *L. curvatus* 54M16 against *L. innocua* C6 after 28 days of storage at 4 °C. A: WPC, control film prepared without *Lb. curvatus 54M16* and WPM, film prepared adding *Lb. curvatus 54M16* and m-MRS broth; B: WPC, control film prepared without *Lb. curvatus* 54M16 and WPH, film prepared adding *Lb. curvatus* 54M16 and distilled water; plates with indicator strain were incubated at 30 °C for 24 h, at 10 °C for 72 h and at 4 °C for 20 days.

Table 6
Antimicrobial activity of WP-based films against *L. innocua* C6 on TSA during storage at 4 °C.

Time (day)	^{a}Log CFU/cm^2 of *L. innocua*		^{a}Log CFU/cm^2 of *L. curvatus* 54M16
	WPM^{b}	WPC^{c}	WPM^{b}
0	5.3 ± 0.2^{Aa}	5.25 ± 0.02^{Aa}	7.55 ± 0.08
1	3.62 ± 0.04^{Ab}	5.3 ± 0.1^{Ba}	7.67 ± 0.03
7	2.13 ± 0.04^{Ac}	6.37 ± 0.03^{Bb}	7.7 ± 0.1
15	1.5 ± 0.1^{Ad}	6.49 ± 0.02^{Bb}	7.77 ± 0.02
28	$<1^{Ae}$	6.42 ± 0.05^{Bbc}	7.82 ± 0.05

a: the values are the means ± SD obtained from duplicate plates of two independent trials; b: WPM, film prepared adding *Lb. curvatus* 54M16 and m-MRS broth; c: WPC, control film prepared without *Lb. curvatus* 54M16. Means within a row with the same capital letter are not significantly different (P > 0.01); means within a column with the same lower case letter are not significantly different (P > 0.01).

L. curvatus count remained stable after the contact of the WPM film with the surface of cooked ham during the 28 days of storage at 4 °C (data not shown). Counts of *L. innocua* are reported in Table 7. The films with *L. curvatus* resulted in a reduction of the microorganism by about 2 log cycles compared to the control films where *L. innocua* increased from 5.77 to 6.47 log CFU/cm^2 at the end of storage period. Similar results were reported by Gialamas et al. (2010) for NaCas films with *L. sakei* assayed against *L. monocytogenes* on fresh beef. Concha-Meyer at al. (2011) found that alginate films with two bacteriocin-producing lactic acid bacteria had a bacteriostatic effect on *L. monocytogenes* on vacuum packed cold-smoked salmon over a period of 28 days.

In the present work differences in antagonistic activity were observed among WPM film by in vitro and in situ study although the film developed showed promising antilisterial activity.

4. Conclusion

Whey protein/gelatin/inulin films can act as effective carriers of LAB in order to be used as bioactive film. The viability of LAB and its antimicrobial properties depends on the nutrient availability. Films obtained in presence of a modified MRS broth showed good LAB viability and antimicrobial properties during storage time. LAB affected the structure of the film with a positive impact on the mechanical properties. In particular, the presence of LAB decreased the film forming solution viscosity and improved the plasticity of the film. LAB had also an impact on the appearance of the film that resulted opaquer. However, LAB had no effect on barrier properties. The use of the bioactive films against *L. innocua* C6, inoculated on laboratory media and on food model systems, resulted in a significant inhibition of the pathogen compared to the control samples. The above results indicate that whey protein-based films carrying a *L. curvatus* 54M16 strain can be used as an effective and alternative packaging technology for improving food safety. Further investigations will be performed to demonstrate the impact of the bioactive film on food shelf life.

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Table 7
L. innocua counts of cooked ham covered with WP-based films during storage at 4 °C.

Time (day)	^{a}Log CFU/cm^2 of *L. innocua*	
	WPM^{b}	WPC^{c}
0	5.8 ± 0.2^{Aa}	5.77 ± 0.05^{Aa}
1	5.40 ± 0.01^{Ab}	5.91 ± 0.01^{Bb}
7	5.39 ± 0.05^{Ab}	5.92 ± 0.02^{Bb}
15	5.1 ± 0.1^{Ac}	6.53 ± 0.09^{Bc}
28	4.62 ± 0.03^{Ae}	6.47 ± 0.03^{Bc}

a: the values are the means ± SD obtained from duplicate plates of two independent trials; b: WPM, film prepared adding *Lb. curvatus* 54M16 and m-MRS broth; c: WPC, control film prepared without *Lb. curvatus* 54M16. Means within a row with the same capital letter are not significantly different (P > 0.05); Means within a column with the same lower-case letter are not significantly different (P > 0.05).

Conflicts of Interest Statement

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

References

Pereira, J. O., Soares, J., J P Monteiro, M., Gomes, A., & Pintado, M. (2018). Impact of whey protein coating incorporated with Bifidobacterium and Lactobacillus on sliced ham properties. *Meat Science, 139*(January), 125–133. https://doi.org/10.1016/j.meatsci.2018.01.016.

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science & Emerging Technologies, 3*, 113–126.

Bekhit, M., Arab-Tehrany, E., Kahn, C. J. F., Cleymand, F., Fleutot, S., Desobry, S., et al. (2018). Bioactive films containing alginate-pectin composite microbeads with lactococcus lactis subsp. Lactis: Physicochemical characterization and antilisterial activity. *International Journal of Molecular Sciences, 19*(2), 1–12. https://doi.org/10.3390/ijms19020574.

Benbettaïeb, N., Karbowiak, T., & Debeaufort, F. (2019a). Bioactive edible films for food applications : Influence of the bioactive compounds on film structure and properties. *Critical Reviews in Food Science and Nutrition*, 1–17. https://doi.org/10.1080/10408398.2017.1393384, 0(0).

Benbettaïeb, N., Karbowiak, T., & Debeaufort, F. (2019b). Bioactive edible films for food applications:Influence of the bioactive compounds on film structure and properties. *Critical Reviews in Food Science and Nutrition, 59*(7), 1137–1153. https://doi.org/10.1080/10408398.2017.1393384.

Blanco Massani, M., Morando, P. J., Vignolo, G. M., & Eisenberg, P. (2012). Characterization of a multilayer film activated with Lactobacillus curvatus CRL705 bacteriocins. *Journal of the Science of Food and Agriculture, 92*(6), 1318–1323. https://doi.org/10.1002/jsfa.4703.

Bolívar-monsalve, J., Ramírez-toro, C., & Bolívar, G. (2019). Trends in Food Science & Technology Mechanisms of action of novel ingredients used in edible fi lms to preserve microbial quality and oxidative stability in sausages - a review. *Trends in Food Science & Technology, 89*(December 2018), 100–109. https://doi.org/10.1016/j.tifs.2019.05.011.

Bruno, M., Giancone, T., Torrieri, E., Masi, P., & Moresi, M. (2008). Engineering properties of edible transglutaminase cross-linked caseinate-based films. *Food and Bioprocess Technology, 1*(4). https://doi.org/10.1007/s11947-007-0031-0.

Calva-Estrada, S. J., Jiménez-Fernández, M., & Lugo-Cervantes, E. (2019). Protein-based films: Advances in the development of biomaterials applicable to food packaging. *Food Engineering Reviews*, 78–92. https://doi.org/10.1007/s12393-019-09189-w, 2019.

Casaburi, A., Di Martino, V., Ferranti, P., Picariello, L., & Villani, F. (2016). Technological properties and bacteriocins production by Lactobacillus curvatus 54M16 and its use as starter culture for fermented sausage manufacture. *Food Control, 59*, 31–45. https://doi.org/10.1016/j.foodcont.2015.05.016.

Coltelli, M. B., Wild, F., Bugnicourt, E., Cinelli, P., Lindner, M., Schmid, M., et al. (2016). State of the art in the development and properties of protein-based films and coatings and their applicability to cellulose based products: An extensive review. *Coatings, 6*(1), 1–59. https://doi.org/10.3390/coatings6010001.

Concha-Meyer, A., Schöbitz, R., Brito, C., & Fuentes, R. (2011). Lactic acid bacteria in an alginate film inhibit Listeria monocytogenes growth on smoked salmon. *Food Control, 22*(3–4), 485–489. https://doi.org/10.1016/j.foodcont.2010.09.032.

Ercolini, D., Ferrocino, I., La Storia, A., Mauriello, G., Gigli, S., Masi, P., et al. (2010). Development of spoilage microbiota in beef stored in nisin activated packaging. *Food Microbiology, 27*(1), 137–143. https://doi.org/10.1016/j.fm.2009.09.006.

Fairley, P., Monahan, F. J., Bruce German, J., & Krochta, J. M. (1996). Mechanical properties and water vapor permeability of edible films from whey protein isolate and sodium dodecyl sulfate. *Journal of Agricultural and Food Chemistry, 44*(2), 438–443. https://doi.org/10.1021/jf9505234.

Ferrocino, I., Storia, A. L., Torrieri, E., Musso, S. S., Mauriello, G., Villani, F., et al. (2013). Antimicrobial packaging to retard the growth of spoilage bacteria and to reduce the release of volatile metabolites in meat stored under vacuum at 1°C. *Journal of Food Protection, 76*(1). https://doi.org/10.4315/0362-028X.JFP-12-257.

Galietta, G., Di Gioia, L., Guilbert, S., & Cuq, B. (1998). Mechanical and thermomechanical properties of films based on whey proteins as affected by plasticizer and crosslinking agents. *Journal of Dairy Science, 81*(12), 3123–3130. https://doi.org/10.3168/jds.S0022-0302(98)75877-1.

García-argueta, I., Dublán-garcía, O., & Quintero-salazar, B. (2013). Effect of lactic acid bacteria on the textural properties of an edible film based on whey. *Inulin and Gelatin, 12*(19), 2659–2669. https://doi.org/10.5897/AJB2013.12134.

Gialamas, H., Zinoviadou, K. G., Biliaderis, C. G., & Koutsoumanis, K. P. (2010). Development of a novel bioactive packaging based on the incorporation of Lactobacillus sakei into sodium-caseinate films for controlling Listeria monocytogenes in foods. *Food Research International, 43*(10), 2402–2408. https://doi.org/10.1016/j.foodres.2010.09.020.

Giancone, T., Torrieri, E., Pierro, P. D., Cavella, S., Giosafatto, C. V. L., & Masi, P. (2011). Effect of surface density on the engineering properties of high methoxyl pectin-based edible films. *Food and Bioprocess Technology, 4*(7). https://doi.org/10.1007/s11947-009-0208-9.

Giello, M., La Storia, A., De Filippis, F., Ercolini, D., & Villani, F. (2018). Impact of Lactobacillus curvatus 54M16 on microbiota composition and growth of Listeria monocytogenes in fermented sausages. *Food Microbiology, 72*, 1–15. https://doi.org/10.1016/j.fm.2017.11.003.

Iseppi, R., Niederh, S. De, Anacarso, I., Messi, P., & Sabia, C. (2011). *Anti-listerial activity of coatings entrapping living bacteria* †. https://doi.org/10.1039/c1sm05650f, 8542–8548.

Kanmani, P., & Lim, S. T. (2013). Development and characterization of novel probiotic-residing pullulan/starch edible films. *Food Chemistry, 141*(2), 1041–1049. https://doi.org/10.1016/j.foodchem.2013.03.103.

Kumariya, R., Kumari, A., Rajput, Y. S., Sood, S. K., & Akhtar, N. (2019). Microbial Pathogenesis Bacteriocins : Classi fi cation , synthesis , mechanism of action and resistance development in food spoilage causing bacteria. *Microbial Pthogenesis, 128* (January), 171–177. https://doi.org/10.1016/j.micpath.2019.01.002.

La Storia, A., Ferrocino, I., Torrieri, E., Di Monaco, R., Mauriello, G., Villani, F., et al. (2012). A combination of modified atmosphere and antimicrobial packaging to extend the shelf-life of beefsteaks stored at chill temperature. *International Journal of Food Microbiology, 158*(3), 186–194. https://doi.org/10.1016/j.ijfoodmicro.2012.07.011.

Lago, M. A., Sendón, R., de Quirós, A. R.-B., Sanches-Silva, A., Costa, H. S., Sánchez-Machado, D. I., et al. (2014). Preparation and characterization of antimicrobial films based on chitosan for active food packaging applications. *Food and Bioprocess Technology, 7*(10). https://doi.org/10.1007/s11947-014-1276-z.

Léonard, L., Gharsallaoui, A., Ouaali, F., Degraeve, P., Waché, Y., Saurel, R., et al. (2013). Preferential localization of Lactococcus lactis cells entrapped in a caseinate/alginate phase separated system. *Colloids and Surfaces B: Biointerfaces, 109*, 266–272. https://doi.org/10.1016/j.colsurfb.2013.03.005.

Mauriello, G., De Luca, E., La Storia, A., Villani, F., & Ercolini, D. (2005). Antimicrobial activity of a nisin-activated plastic film for food packaging. *Letters in Applied Microbiology, 41*(6), 464–469. https://doi.org/10.1111/j.1472-765X.2005.01796.x.

Menard, K. P. (1999). *Dynamic mechanical analysis: A practical introduction* (2nd ed.).

Pelegrine, D. H. G., & Gasparetto, C. A. (2005). Whey proteins solubility as function of temperature and pH. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology, 38*(1), 77–80. https://doi.org/10.1016/j.lwt.2004.03.013.

Pereira, J. O-, Soares, J., Sousa, S., Madureira, A. R., Gomes, A., & Pintado, M. (2016). Edible films as carrier for lactic acid bacteria. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology, 73*, 543–550. https://doi.org/10.1016/j.lwt.2016.06.060.

Pereira, J. O., Soares, J., Costa, E., Silva, S., & Gomes, A. (2019). *Characterization of edible films based on alginate or whey protein incorporated with Bifidobacterium*.

Perone, N., Torrieri, E., Cavella, S., & Masi, P. (2014). Effect of rosemary oil and HPMC concentrations on film structure and properties. *Food and Bioprocess Technology, 7* (2). https://doi.org/10.1007/s11947-012-1044-x.

Quintavalla, S., & Vicini, L. (2002). *Antimicrobial food packaging in meat industry, 62*, 373–380.

Sánchez-gonzález, L., Iván, J., Saavedra, Q., & Chiralt, A. (2013). Food Hydrocolloids Physical properties and antilisterial activity of bioactive edible fi lms containing Lactobacillus plantarum. *Food Hydrocolloids, 33*(1), 92–98. https://doi.org/10.1016/j.foodhyd.2013.02.011.

Sánchez-González, L., Quintero Saavedra, J. I., & Chiralt, A. (2014). Antilisterial and physical properties of biopolymer films containing lactic acid bacteria. *Food Control, 35*(1), 200–206. https://doi.org/10.1016/j.foodcont.2013.07.001.

Shoaib, M., Shehzad, A., Omar, M., Rakha, A., Raza, H., Sharif, H. R., et al. (2016). Inulin: Properties, health benefits and food applications. *Carbohydrate Polymers, 147* (April), 444–454. https://doi.org/10.1016/j.carbpol.2016.04.020.

Siripatrawan, U., & Harte, B. R. (2010). Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocolloids, 24* (8), 770–775. https://doi.org/10.1016/j.foodhyd.2010.04.003.

Soukoulis, C., Behboudi-Jobbehdar, S., Yonekura, L., Parmenter, C., & Fisk, I. D. (2014). Stability of Lactobacillus rhamnosus GG in prebiotic edible films. *Food Chemistry, 159*, 302–308. https://doi.org/10.1016/j.foodchem.2014.03.008.

Torrieri, E., Cavella, S., & Masi, P. (2015). Effect of Rosemary Oil and an emulsion of essential oils on structure and physical properties of chitosan film. *Chemical Engineering Transactions, 43*. https://doi.org/10.3303/CET1543005.

Volpe, S., Cavella, S., Masi, P., & Torrieri, E. (2017). Effect of solid concentration on structure and properties of chitosan-caseinate blend films. *Food Packaging and Shelf Life, 13*. https://doi.org/10.1016/j.fpsl.2017.07.002.

Yildirim, S., Röcker, B., Pettersen, M. K., Nilsen-Nygaard, J., Ayhan, Z., Rutkaite, R., et al. (2018). Active packaging applications for food. *Comprehensive Reviews in Food Science and Food Safety, 17*(1), 165–199. https://doi.org/10.1111/1541-4337.12322.