Antimicrobial and physicochemical characterization of whey protein concentrate edible films incorporated with liquid smoke

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ABSTRACT

The incorporation of commercial liquid smoke (LS) to edible films was investigated for the first time. The objective of this investigation was to characterize whey protein concentrate (WPC)-based edible films incorporated with LS. According to the bactericidal activity of LS against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Listeria monocytogenes* in liquid medium, WPC-based films incorporated with 0, 5, 10, and 15% (v/v) LS were prepared. Inhibition zone in solid media, thickness, transparency, color and mechanical properties of the films were analyzed. Films including LS were effective to prevent growth of *L. monocytogenes* in the agar diffusion test. Analyzing color parameters, the incorporation of LS into films caused a decrease in L* and an increase in both a* and b*. However, these sensory changes were not detrimental for their potential use in food applications. Noticeably, tensile strength and elongation tend to increase when LS was added into films formulation. Depending on its content, different protein-LS interactions could be generated, positively affecting the mechanical properties of films. In conclusion, WPC-based edible films incorporated with LS may be suitable for being applied to food surfaces and useful to prevent the superficial growth of the globally recognized high-risk foodborne pathogen *Listeria monocytogenes*.

Keywords: whey protein films, liquid smoke, antimicrobial properties, physicochemical characterization.

1. Introduction

Packaging is widely used for protection of food quality, thereby ensuring hygiene and extending the shelf life of perishable items, especially those susceptible to oxidative and microbiological deterioration (Ahmad, Benjakul, Prodpran, & Agustini 2012). Nowadays, packaging research is receiving a considerable attention due to the development of eco-friendly materials made from natural polymers often from waste products from agriculture, livestock raising, or fishing. Such polymers may be protein, lipid, or polysaccharide based and could be used to formulate edible films which can be an integral part of the food, or act as a complementary packaging thereby reducing the level of material discarded to the environment (Gómez-Estaca, Giménez, Montero, & Gómez-Guillén, 2012). In particular, the use of whey proteins to manufacture films has received a great deal of attention since these proteins allow upgrade of a cheese-making effluent, and possess interesting mechanical and barrier properties (Ramos *et al*., 2013).

The use of edible films incorporating plant extracts, essential oils, antioxidants, colorants, flavors, fortifying nutrients or spices could offer some additional benefits such as the improve of nutritional and/or organoleptic characteristics of the product to which it is applied (Bourtoom, 2008; Falguera, Quintero, Jiménez, Aldemar Muñoz, & Ibarz, 2011). Edible films can also serve as carriers for a wide range of GRAS additives, including compounds with antimicrobial properties that can extend product shelf life and reduce the risk of growth of pathogenic and food spoilage organisms on food surfaces. Several antimicrobial edible films have been developed to minimize growth of microorganisms which may contaminate the surface of cooked ready-to-eat foods after processing. Some of the more commonly used preservatives and antimicrobials includes organic acids such as benzoates, propionates or sorbates, among others (Appendini & Hotchkiss, 2002; Suppakul, Miltz, Sonneveld, & Bigger, 2003, Cagri, Ustunol, & Ryser, 2004; Kuorwel, Cran, Sonneveld, Miltz, & Bigger,

2011; Kraśniewska & Gniewosz, 2012; Rocha, Ferreira, Souza, & Prentice, 2013; Pérez, Soazo, Balagué, Rubiolo, & Verdini, 2014).

The greater consumer awareness and concern regarding synthetic chemical additives had improved the popularity of foods preserved with natural additives. Smoke flavourings have been used for some 30 years as preservatives and aromatizers of meat and fish (Martinez, Salmerón, Guillén, & Casas, 2007). Many authors have studied the antimicrobial activity (Vitt, Himelbloom, & Crapo, 2001; Suñen, Aristimuno, & Fernandez-Galian, 2001; Holley & Patel, 2005; Milly, Toledo & Ramakrishnan, 2005; Gedela, Escoubas, & Muriana, 2007a; Gedela, Gamble, Macwana, Escoubas, & Muriana, 2007b; Martin *et al*., 2010; Morey, Bratcher, Singh, & McKee, 2012), antioxidant effects (Coronado, Trout, Dunsheac, & Shah, 2002; Huang, Chang, Sung, Vong, & Wang, 2011) and organoleptic properties (Guillén & Ibargoitia, 1998; Ojeda, Bárcenas, Pérez-Elortondo, Albisu, & Guillén, 2002) of smoke flavourings. Liquid smoke (LS) is a solution of natural wood smoke flavors produced by condensing wood smoke created by the controlled, minimal oxygen pyrolysis of sawdust or wood chips (Lingbeck *et al*., 2014). Some authors studied the composition of LS preparations and the sensory properties of various smoke fractions and isolated compounds (Montazeri, Oliveira, Himelbloom, Leigh, & Crapo, 2013; Pino, 2014). LS was reported to offer many advantages over traditional smoking in a kiln, namely ease of application, speed and product uniformity. Commercial LS products contain phenols, carbonyl compounds and acetic acid, which are bactericidal at relatively low concentrations. LS can inactivate both food spoilage organisms and common food-borne pathogens, including *Escherichia coli*, *Salmonella sp*., *Staphylococcus aureus*, and *Listeria monocytogenes* (Vitt *et al*., 2001; Holley & Patel, 2005; Milly *et al*., 2005; Gedela *et al*., 2007a,b; Martin *et al*., 2010; Morey *et al*., 2012; Lingbeck *et al*., 2014). Based in all these evidences, antimicrobial, antioxidant, coloring, and flavouring properties, makes LS a potentially attractive additive for edible films. Moore & Moore (1998)

reported the incorporation of LS into edible films made from different polymers such as hydroxymethyl propyl cellulose, casein, carrageenan, or whey proteins; but films characteristics were not fully analyzed. Taking into account that edible films are heterogeneous in nature, physicochemical properties can be seriously affected due to the interactions between proteins and added components (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Salgado, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2013).

In the present work, commercial liquid smoke (LS), a widely used surface antimicrobial, was incorporated for the first time to edible films. Therefore, the objective of this investigation was to characterize the microbiological and physicochemical aspects of WPC-based edible films formulated with the addition of different concentrations of commercial LS.

2. Materials and methods

2.1. Materials

Whey protein concentrate (WPC) 80% (Arla Food Ingredients S.A, Martinez, Argentina) was used as the main component to prepare film forming solutions. Glycerol (Gly) (Cicarelli, San Lorenzo, Argentina) was added as plasticizer. Liquid Smoke (LS) (San Giorgio, Buenos Aires, Argentina) was incorporated as additive. Trypticase Soy Broth (TSB), Trypticase Soy Agar (TSA), and Oxford Modified Agar Base culture media were purchased from Britania (Buenos Aires, Argentina). All other reagents were of high-purity grade and used as received.

2.2. Characterization of commercial LS

LS was characterized according to its color, total solid content, pH, titratable acidity, total phenol derivatives, total carbonyl derivatives, particle size distribution, and ζ-potential. pH was recorded with a pH-meter (Metrohm 713, Metrohm Ltd., Herisau, Switzerland). Total

124 solid content was calculated as the remaining weight of LS after drying at 105 ºC and was
125 expressed as percentage of the liquid sample. For titratable acidity (as % acetic acid) LS was
126 diluted in deionized water and titrated to pH 8.3 using 0.1 N NaOH (Montazeri *et al.*, 2012).
127 Total phenol derivatives were estimated through a modification of the Folin Ciocalteu method
128 and were expressed as mg of gallic acid equivalent (GAE) kg^{-1} (Chun, Kim, Smith,
129 Schroeder, Han, & Lee, 2005). Total carbonyl derivatives were determined by a method based
130 on their reaction with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone
131 (ASTM-E411) and were expressed as mg of carbonyl kg^{-1}. Particle size distribution and ζ-
132 potentials of LS aqueous solutions adjusted to the pH value achieved in film forming
133 solutions (5, 10, and 15% LS, pH 5.7, 5.2, and 4.8, respectively) were measured using a
134 particle analyzer (Horiba Nano SZ-100, Horiba Scientific, Kyoto, Japan). Since the LS
135 manufacturer reported the presence of potassium sorbate, this preservative was quantified by
136 HPLC. A chromatography column C18, 25 cm x 4.6 cm, 5 μm (Supelco, PA, USA) and an
137 UV/VIS detector (Gilson 151, Gilson, WI, USA) were used. The determination was carried
138 out at room temperature operating with a flow rate of 1 mL min^{-1} and isocratic elution with
139 sodium acetate (pH 4.2)/ acetonitrile in relation 80/20 as mobile phase. All above
140 determinations were performed in triplicate. LS characteristics are showed in Table 1.
141
142 **2.3. Inhibitory activity of LS in liquid media**
143 Minimum inhibitory concentration (MIC) of LS against *Escherichia coli* O157:H7 ATCC
144 43895, *Staphylococcus aureus* ATCC 43300, *Salmonella* Typhimurium ATCC 14028, and
145 *Listeria monocytogenes* ATCC 19115 was estimated according to the National Committee for
146 Clinical Laboratory Standards-recommended macrodilution broth method (2009) as described
147 by Pérez *et al.* (2014). Briefly, an overnight culture of each bacterial strain in TSB was
148 adjusted to McFarland 0.5 standard in saline solution (approximately 5 x 10^7CFU/mL) and

used as test inoculum. One mL of the bacterial inoculum was added and mixed with 1 mL of each LS solution in TSB (pH 5.5). The medium condition (pH 5.5) was selected in order to provide a pH value similar to those reported for foods like cheeses and meats (Casp & Abril, 2003), but considering the acidity limit for pathogens growth and survival (Kaper, Nataro, & Mobley, 2004). The LS concentrations evaluated were 0.625, 1.25, 2.5, 5.0, and 10% (v/v). A control tube without LS was inoculated to test microbial growth and a tube containing only broth medium was evaluated to discard possible contaminations. All tubes were incubated during 48 h at 37 °C. The MIC values were estimated as the lowest concentration of LS that completely inhibits growth of the microorganism tested as can be detected by the unaided eye (*i.e.*, no turbidity after incubation was an indicative of growth inhibition). Growth control tubes to assess MIC end points were also evaluated.

To evaluate the minimum bactericide concentration (MBC) 100 µL of negative tubes (*i.e.* showing no turbidity in the MIC determination assays) were sprayed into Petri dishes containing TSA (pH 7.2). The inoculated plates were incubated at 37 ºC during 48 h and the MBC was determined as the lowest concentration of LS yielding colony counts less than 0.1% of the initial inoculum.

2.4. Preparation of film-forming solutions

Edible films (casting solution 11.5% total solids) were obtained with a modification of the method described by Soazo, Rubiolo & Verdini (2011). Briefly, WPC and Gly (in proportion WPC/Gly 3:1 w/w dry solid basis) were dissolved in distilled water. After mixing, the solution was heated at 90 ºC for 30 min in a water bath (TDS-40, TecnoDalvo, Santa Fe, Argentina) to achieve whey proteins denaturation. Then, whey protein solutions were homogenized (5 min; 20000 rpm) with an Omni GLH homogenizer (Omni International Inc., Kennesaw, USA). Finally, solutions were degassed by sonication (Cole-Parmer 8890E-MT,

Cole-Parmer, Buenos Aires, Argentina) during 60 min and LS was added at 0, 5, 10, and 15% (v/v). The pH of the film forming solutions were 6.5, 5.7, 5.2, and 4.8 corresponding to solutions with 0%, 5%, 10%, and 15% LS, respectively. The pH decrease of the film forming solutions due to the acidity of LS may overlap with the effect of most LS compounds, considering that smoke condensates mainly contains phenols, carbonyls, and organic acids (compounds that have acid-base behavior). Thus, to discriminate if film properties when LS was added, are related only to pH variations or to the combination of pH and compounds present in LS itself, pH Control films (*i.e.*, without LS) were prepared adjusting the pH of the solution to 5.7, 5.2 and 4.8 with 1.0 N HCl. Then, the films obtained from such solutions were named as C 5%, C 10% and C 15%, respectively.

2.5. Film formation

Films were prepared by pipetting 8 g of the degassed solutions on 90 mm diameter disposable polyethylene Petri dishes. Films were dried on a leveled surface in an environmental chamber (SCT Pharma, Temperley, Argentina) at 25 °C and constant relative humidity (RH, 58%). After drying, films were removed from the plates and were conditioned in the environmental chamber set at 25 °C and 58% RH for 24 h. Films used in the different tests were selected based on the lack of physical defects such as cracks, bubbles and holes.

2.6. Inhibition zone assay in agar media

The antimicrobial activity of WPC-based edible films incorporated with LS was evaluated by the inhibition zone assay in solid media as described by Pérez *et al*. (2014). Briefly, films were aseptically cut in 12 mm diameter discs using a sterile cork borer. Then, discs were aseptically transferred to pour plates containing 10 mL of Oxford modified agar (for *L. monocytogenes*) or TSA (for *E. coli*, *S. aureus*, and *S.* Typhimurium) both media acidified to

pH 5.5 with 1.0 N HCl, which had been previously seeded with a bacterial suspension adjusted to 0.5 McFarland standard in saline solution. After overnight incubation (~18 h) at 37 ºC the diameter of the inhibition zone represented by a clear area of non-growth around the film disc was measured perpendicularly using a caliper. Films without LS were used as negative controls of the assay. Experiments were performed in triplicate. The medium condition (pH 5.5), as explained previously, provided a pH value similar to those reported for foods like cheeses and meats, but considering the acidity limit for pathogens growth and survival.

2.7. Film thickness

Film thickness was measured with a digital micrometer (Schwyz, China). For each film, nine thickness measurements were taken. Averaged values were calculated and used in next studies.

2.8. Optical properties

2.8.1. Transparency

The visible light barrier properties were measured on films at selected wavelengths (in the 400-800 nm range), using a spectrophotometer (Jasco V-530, Jasco International, Tokyo, Japan). Film samples were cut into rectangular pieces (10 mm x 30 mm) and placed on the internal side of a spectrophotometer cell. The relative transparency of films was measured at 560 nm as described by Pérez *et al*. (2014). Five replicates of each film were tested. Transparency (%) was calculated as the percentual relationship between the light intensity with the specimen in the beam and the light intensity with no specimen in the beam.

2.8.2. Color analysis

Films were cut in 12 mm diameter discs using a cork borer and were used to obtain the digital images. A wooden box constructed according to the design described in Mendoza and Aguilera (2004), with some modifications, was used. Samples were illuminated using 4 fluorescent lamps (Osram, Biolux, Natural Daylight, 18W/965, München, Germany) with a color temperature of 6500 K (*D65*, standard light source commonly used in food research) and a color-rendering index Ra of 95%. Additionally, electronic ballast and an acrylic light diffuser ensured uniform illumination system. Discs were photographed employing a digital camera (Nikon P 7100, Nikon, Jakarta, Indonesia) on a matte white background using the following camera settings: manual mode with lens aperture at f = 8, time of exposition 1/50, no flash, ISO sensibility 400, maximum resolution, and storage mode in RAW format.

The International Color Consortium (ICC) profile was used and images were processed to obtain L*, a*, and b* parameters average values (considering the whole sample) using Photoshop® (Adobe Systems Inc., Mountain View, USA), as explained in Soazo *et al.* (2015).

2.9. Tensile test

Tensile test was carried out using a Multi Test 2.5-D motorised test frame (Mecmesin, VA, USA) equipped with a 25 N digital force gauge. Films were cut into strips (7 mm x 60 mm) using a scalpel. Strip ends were mounted with double sided tape and rectangles of 30 mm wide and 10 mm length of cardstock. The exposed film strip length, between cardstock ends, was 30 mm. The cardstock pads were placed at the ends of film strips to prevent tearing and slippage in the testing device (Shellhammer & Krochta, 1997). Probes, prepared as explained previously, were conditioned for 1 day at 25 °C and 58% RH. The initial grip distance and crosshead speed were 30 mm and 0.05 mm/s, respectively. The parameters obtained from

249 stress-strain curves were: tensile strength (TS) calculated by dividing the peak load by the
250 cross sectional area (thickness of film × 7 mm) of the initial film, and elongation (E)
251 calculated as the percentile of the change in the length of specimen respect to the original
252 distance between the grips 30 mm (Han, Seo, Park, Kim, & Lee, 2006). Five replications were
253 performed.
254
255 **2.10. Statistical analysis**
256 Statistical analysis was performed using Statgraphics Plus 5.1 program (Statpoint
257 Technologies, Inc., Warrenton, USA). Analysis of variance (ANOVA) was used to analyze
258 data and when the effect of the factors under study was significant the test of multiple ranks
259 honestly significant difference (HSD) of Tukey was applied. A significance level of $\alpha = 0.05$
260 was used.
261
262 **3. Results and discussion**
263
264 **3.1. Inhibitory activity of LS**
265
266 **3.1.1. Liquid media**
267 To examine the antimicrobial properties of the commercial LS, *E. coli*, *S. aureus*, *S.*
268 Typhimurium, and *L. monocytogenes*, which are very significant pathogens in the food
269 industry, were tested. The MIC and MBC values for LS were 5% v/v for all strains, indicative
270 of a bactericidal activity, showing that the LS used in the present work was equally effective
271 for all strains analyzed. These data were used to define the concentration of LS subsequently
272 incorporated into edible film formulations. Milly (2003) discussed the difficulty of identifying
273 the mechanism and compounds responsible for the microbial inhibition of LS. Consequently,

274 the efficacy of smoke condensates with regard to antimicrobial potential depends on the
275 concentration of phenols, carbonyls, and organic acids and the test microorganism (Milly *et*
276 *al*., 2005; Gedela *et al*., 2007a,b; Montazeri *et al*., 2012; Lingbeck *et al*., 2014). The
277 characterization of the LS used in this investigation confirmed the presence of carbonyl and
278 phenolic compounds (Table 1).
279
280 **3.1.2. Inhibition zone assay in agar media**
281 Table 2 shows that films containing LS were only effective to inhibit growth of *L.*
282 *monocytogenes*, whereas failed to restrain *E. coli*, *S.* Typhimurium, and *S. aureus*. Film discs
283 with LS contents of 5%, 10%, and 15% inhibited *L. monocytogenes* with inhibition zones
284 ranging from 1.7 ± 0.6 to 5.5 ± 0.5 mm, being inhibition zones dependent on LS content. 0%
285 films and pH Control films were always non-inhibitory. Interestingly, despite the lack of an
286 inhibition zone around the film discs incorporating LS observed for *E. coli*, *S. aureus*, and *S.*
287 Typhimurium, no evidence of microbial growth over the discs was observed.
288 Although the antimicrobial activity of LS depends on the concentration of phenols, carbonyls,
289 and organic acids and the test microorganism, and considering that in liquid media al strains
290 analyzed showed equal MIC and MBC values, results were strain-dependent when LS was
291 incorporated into WPC-based films. When LS activity is tested in liquid media, compounds
292 with antimicrobial activity are free and available to move and react. However, when LS is
293 incorporated into edible films, such compounds may be retained in the film matrix, and thus
294 their antimicrobial activity could not be evidenced due to some restrictions in the diffusion of
295 chemicals form WPC films to solid media. Carbonyl and phenolic compounds may strongly
296 interact with milk proteins at film matrix (Damodaran & Kinsella, 1980; Ozdal, Capanoglu, &
297 Altayb, 2013). Recently, Zhang *et al*. (2014) reported that phenolic acids interact with the
298 structural subunits of the two most abundant proteins in milk whey, α-lactalbumin and β-

299 lactoglobulin, thus altering protein conformation. Therefore, it is feasible that LS carbonyl
300 and phenolic compounds with antimicrobial properties could be retained by the WPC film
301 matrix. In addition, the diffusion of the different compounds with antimicrobial activity
302 present in LS may be related to both particle size and interaction with WPC matrix. Overall,
303 *L. monocytogenes* may be more sensitive than the other strains to some compounds released
304 from the films.
305 Moreover, as can be seen in Table 1, the low concentration of potassium sorbate added by the
306 manufacturer into the original LS product would not be responsible for the observed
307 antimicrobial activity of films, since at the maximum concentration of LS incorporated (15%
308 v/v) the final concentration of potassium sorbate in the films was approximately 0.01%.
309 Previous results from our group showed no evidence of antimicrobial activity against the four
310 strains analyzed in the present study for WPC-based films formulated with the incorporation
311 of 0.25% potassium sorbate (Bessone, 2015).
312

313 **3.2. Optical properties**

314 The addition of LS affected transparency and color of WPC-based films, as can be seen in
315 Figures 1 and 2. Transparency decreased when LS was incorporated into film formulation,
316 and this effect was observed at all LS concentrations evaluated. These results could be related
317 with water-soluble organic carbon compounds from wood combustion present in LS that
318 absorbs visible light (Chen & Bond, 2010). Moreover, commercial LS contains caramel color
319 (E150) as a soluble food coloring that absorbs visible light at 560 nm (JECFA, 2011). Further,
320 the addition of LS decreased the pH of the film forming solutions, thus our observations
321 agreed with Pérez *et al*. (2014), who reported that transparency decrease in WPC-based films
322 at acidic pH could be explained due to a partial protein precipitation when the pH of the film
323 forming solutions is close to the isoelectric point of whey proteins (pI ~ 5). Thus, pH decrease

caused by LS addition in the film-forming solutions also contributed to the observed decrease in films transparency.

In practical applications, color influences the appearance of edible films which in turn condition consumer choice. The incorporation of LS into film formulation also affected the color of the films decreasing the L* values (Fig. 2). These results reflect the fact that WPC-based films became darker when LS was included in the film formulation. These changes were expected because of the dark brown color of LS, which is characteristic of smoked products. Besides, Du, Olsen, Avena-Bustillos, Friedman, and McHugh (2011) reported that the inclusion of phenolic compounds darkened edible films made from apple puree. The addition of LS caused an increase in yellowness and redness for WPC films as indicated by higher a* and b* values when comparing to 0% LS films (Fig. 2). As a result, the characteristic yellowish color of WPC-based films changed to brown with the addition of increasing concentrations of LS at edible films formulation.

3.3. Thickness and mechanical properties

Table 3 shows thickness and parameters obtained from force-deformation curves in tensile test for WPC-based edible films with different concentrations of LS and their respective pH Control films. No significant difference in film thickness was detected among WPC-based edible films containing different LS concentration. Our results were consistent with related reports demonstrating that incorporation of phenolic and carboxylic compounds at various concentrations did not significantly affect the thickness of protein films (Arcan & Yemenicioğlu, 2011; Cheng, Wang, & Weng, 2015), suggesting that phenolic and carbonyls compounds could be distributed in the film matrix without affecting the film thickness.

In reference to mechanical parameters, TS and E tend to increase when LS was added up to LS 10% and tend to decrease when LS was 15%. On the other hand, TS and E parameters

where higher in films with LS compared with each pH Control film; however differences were significant only between LS 10% and C 10%. This fact suggests that different interactions between the protein-based film matrix and LS components should be considered to explain the observed effect. Intermolecular hydrogen bonding between the N-terminal part of whey proteins and phenolic compounds presented in LS could enhance the cross-linkage. This phenomenon was reported by other researchers in edible films containing polyphenols. For example, Sun, Wang, Kadouh and Zhou (2014) demonstrated that the incorporation of gallic acid into chitosan films significantly increased its TS, which could be attributed to the formation of intermolecular hydrogen bonding between the NH_3^+ of the chitosan backbone and the OH^- of gallic acid, an hydrolysable tannin present in substantial amounts in teas and coffees (Chaturvedula & Prakash, 2011). However, these authors reported that when the added concentration of gallic acid was higher, the TS of the resulting films decreased possibly because an excessive dispersion of the phenolic acid in the film which crack the inner structure.

In order to go deeper in the study of these phenomena and taking into account that the LS used in the present work contains suspended nanoparticles, particle size distribution and ζ-potential of LS were studied. Size distribution of LS particles was bimodal, presenting two peaks, one around 22.6 nm and the other at 177.4 nm, on average (Table 1). These particles are generated during the wood combustion in LS industrial production. Laiho *et al.* (2015) showed that average particle size of whey protein dispersions heated at 90 °C for 5 min was 600 nm and larger aggregates with a diameter around 7000 nm were also found to exist. Thus, particles present in LS were smaller than aggregates formed by heating whey protein suspensions. Therefore, LS particles could be included in interstitial spaces between protein aggregates and this phenomenon certainly affected mechanical properties of films. Moreover, LS had a negative ζ-potential at the three concentrations studied (5, 10, and 15% v/v),

374 although the absolute value decreased when the LS content increased (Table 1). So, in film
375 formation, three different situations should be distinguished according to the pH of the
376 environment. When 5% LS was added to film forming solution, the final pH reached 5.7,
377 being higher than the isoelectric point of whey proteins (pI~ 5). Since protein molecules and
378 LS particles were negatively charged, repulsive forces between protein-protein aggregates,
379 protein-LS, and LS-LS particles were the predominant interactions in the system. In film
380 forming solution with 10% LS the final pH reached 5.2, close to the pI, therefore, protein
381 aggregates with approximately zero net charge can get close enough to form strongly bonded
382 structures. Finally, when 15% LS was added to the film forming solution, protein aggregates
383 were positively charged because the final pH reached (pH=4.9) was lower than the pI and
384 repulsive forces between protein-protein and LS-LS and attractive forces among protein and
385 LS particles could be generated. In the first and in the last case, repulsive forces decreased the
386 occurrence of particle associations within the protein matrix and thus contributing to the
387 formation of films with lower TS values.
388
389 **4. Conclusions**
390 WPC-based edible films formulated with the addition of LS were effective to inhibit growth
391 of *L. monocytogenes*. Interestingly, despite the lack of an inhibition zone around the film
392 discs incorporating LS observed for *E. coli*, *S. aureus*, and *S.* Typhimurium, no evidence of
393 microbial growth over the discs was observed. The typical yellowish color of WPC-based
394 films changed to a more attractive brown color, characteristic of smoked products, with the
395 addition of increasing concentrations of LS at edible films formulation. In addition, these
396 color changes masked the lack of transparency when increasing LS concentration at the film
397 formulation. Moreover, WPC-based films with the addition of LS showed a characteristic
398 smoked aroma that may be attractive for consumers, suggesting that these films could be very

promising for food applications. Furthermore, incorporation of LS tend to increase mechanical resistance of WPC-based films, which is a desired aspect for packaging applications because preserves film integrity during processing and handling. Thus, edible films described in this work could be an interesting alternative not only to improve organoleptic aspect of wrapped foods but also quality and safety of food products including dairy products, raw meat, vegetables and seafood, feasible of contamination with *L. monocytogenes*, a recognized high-risk pathogen.

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605

606 **Table 1.** Characterization of commercial liquid smoke.

Color	Dark brown
Total solid content (% w/w)	4.26±0.07
pH	2.54±0.05
Titratable acidity (% acetic acid)	1.6±0.1
Total phenol derivatives (mg GAE kg^{-1})[a]	6528±78
Total carbonyl derivatives (mg kg^{-1})	7848±77
Potassium sorbate (mg/mL)	0.699±0.009
Particle size (nm)[b]	LS 5%: 18.8±1.1 (0.26); 161,1±16,5 (0.68) LS 10%: 26.6±5 (0.30); 177.9±30.7 (0.70) LS 15%: 22.6±2.5 (0.22); 193.2±44.1 (0.78)
ζ-potential (mV)	LS 5%: -29.7±2.3 LS 10%: -20.5±2.3 LS 15%: -12.1±2.9

607 [a] GAE, gallic acid equivalent.

608 [b] Numbers in parentheses refer to relative area of each peak.

609 Data corresponds to mean values and standard deviations of three samples.

610 **Table 2.** Inhibition zone assay of WPC-based edible films with different concentrations of LS

611 in agar media.

Strain	Diameter of inhibition zone (mm)			
	WPC films + LS			
	0%	LS 5%	LS 10%	LS 15%
E. coli ATCC 43895	0[a]	0[a]	0[a]	0[a]
S. aureus ATCC 43300	0[a]	0[a]	0[a]	0[a]
S. Typhimurium ATCC 14028	0[a]	0[a]	0[a]	0[a]
L. monocytogenes ATCC 19115	0[a]	1.7 ± 0.6[b]	3.6 ± 0.9[c]	5.5 ± 0.5[d]

612

613 WPC: whey protein concentrate. LS: liquid smoke.

614 pH Control films were always non-inhibitory.

615 Data corresponds to mean values and standard deviations of five samples.

616 Values with different letters in each column are significantly different ($p < 0.05$).

Table 3. Thickness and parameters derived from tensile test of WPC-based edible films with different concentrations of liquid smoke and their respective pH Control films. TS: tensile strength; E: elongation.

Film	Thickness (mm)	TS (MPa)	E (%)
LS 0%	0.140±0.005^{a}	1.33±0.74^{a}	1.43±0.53^{ab}
LS 5%	0.137±0.008^{a}	1.52±0.37^{ab}	1.70±0.47^{abc}
LS 10%	0.139±0.006^{a}	2.62±0.82^{b}	2.49±0.77^{c}
LS 15%	0.132±0.005^{a}	2.09±0.74^{ab}	2.09±0.41^{bc}
C 5%	0.139±0.014^{a}	1.16±0.40^{a}	1.07±0.24^{a}
C 10%	0.127±0.005^{a}	1.28±0.52^{a}	1.14±0.38^{a}
C 15%	0.140±0.004^{a}	1.40±0.35^{a}	1.51±0.32^{ab}

Data corresponds to mean values and standard deviations of five samples.

Values with different letters in each column are significantly different ($p < 0.05$).

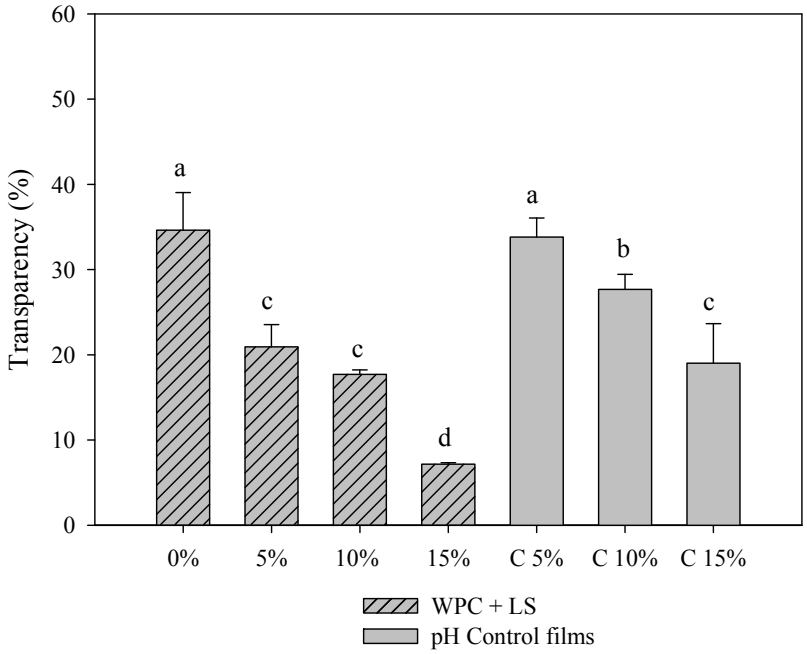
626 F**igure captions**

627

628 **Figure 1.** Transparency of WPC-based edible films with different concentrations of LS and
629 their respective pH Control films. Bars are based on standard deviations. Different letters
630 show significant differences ($p < 0.05$).

631

632 **Figure 2**. Colour parameters of WPC-based edible films with different concentrations of LS
633 and their respective pH Control films. Bars are based on standard deviations. Different letters
634 show significant differences ($p < 0.05$).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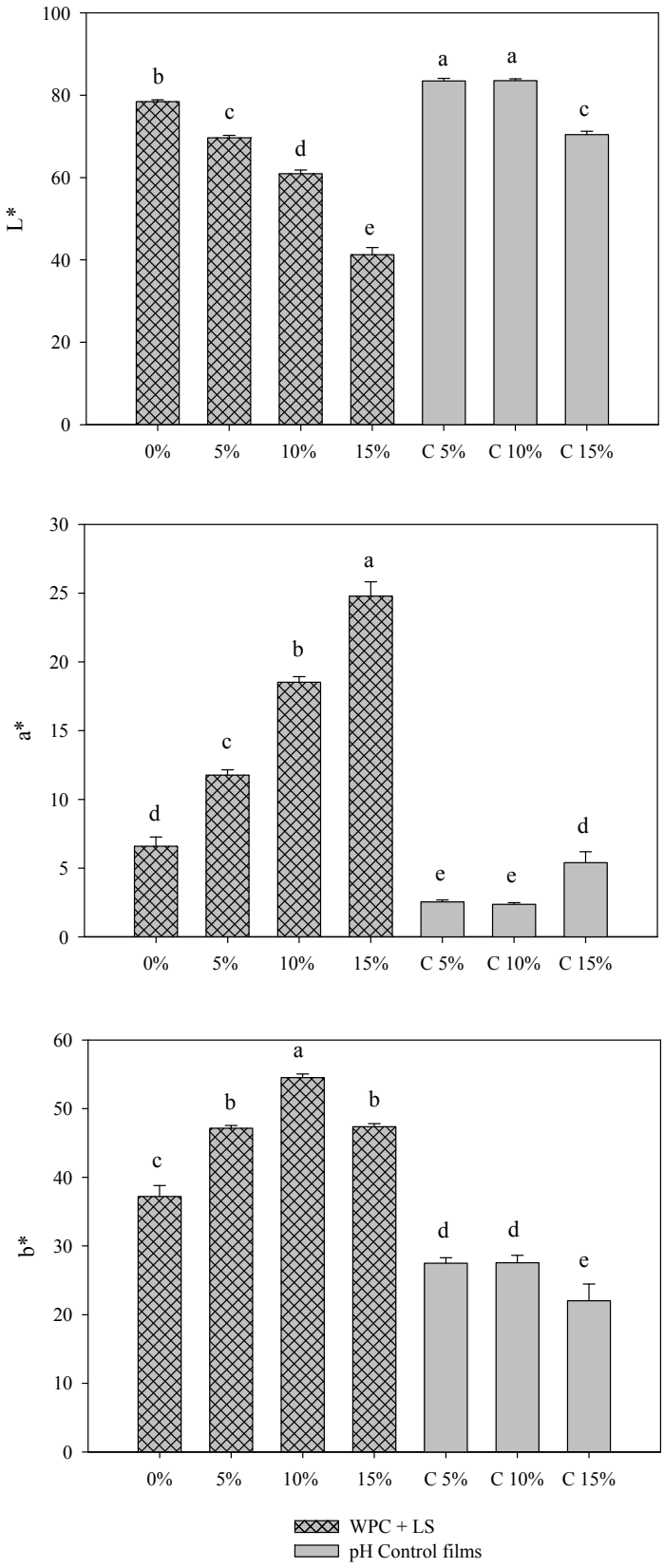
WPC + LS
pH Control films

Figure 1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WPC + LS
pH Control films

Figure 2