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INFLUENCE OF THE PROCESSING METHOD AND ANTIMICROBIAL AGENTS ON PROPERTIES OF STARCH-GELATIN BIODEGRADABLE FILMS.

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Abstract: Biodegradable films based on corn starch (CS), bovine gelatin (BG), glycerol (GL) as a plasticizer, and lysozyme (LZ) or N-α-lauroyl-l-arginine ethyl ester monohydrochloride (LAE) as antimicrobial agents were obtained by both, extension-drying (*casting*) of the aqueous dispersions and melt blending and compression molding. Microstructural analyses revealed the lack of miscibility between CS and BG, which implied polymer phase separation, with the formation of domains rich in each polymer, with different arrangement for casting and melt-blending processes. Thermo-processed films were more permeable to water vapor (60-115%) and oxygen (70-355%), compared to the corresponding casting films and exhibited lower stiffness (50-75%) and resistance to break (17-33%) and greater extensibility (150-190%) than casting films. LAE improved water vapor barrier and reduced oxygen barrier of the both kinds of films, whereas the opposite effect was observed for LZ. Antimicrobial activity against *Listeria innocua* was observed for formulations containing LAE

processed by both casting and compression molding, all of which exhibited a bactericidal effect.

Keywords: Corn starch, bovine gelatin, lysozyme, LAE, *Listeria innocua*.

Introduction

Petroleum-derived synthetic plastics have been traditionally used as packaging materials due to their availability, low cost, good mechanical and barrier properties and their thermo-processing ability. In the food industry, these materials are broadly used as packaging materials to preserve and protect food from physical damage, oxidation and microbial spoilage. However, the accumulation of these non-biodegradable materials is a serious environmental problem while their recycling incurs a high cost. This is why new materials based on biodegradable polymers have been developed in the last few years [1].

Starch and gelatin are biodegradable materials, widely available, low cost and with very good film forming ability[2, 3, 4, 5]. The combination of TPS (thermoplastic starch) with other polymers, such as gelatin, has been pointed out as a way of enhancing film mechanical behavior, leading to films with higher resistance and elongation capacity [6, 7, 8, 9]. Previous works reflected that the starch:gelatin combination (1:1 mass ratio) yielded films with improved mechanical resistance and extensibility [6].

Incorporation of bioactive agents into biodegradable films enhanced their functionality and added value to obtain active packaging materials. These compounds also favour the preservation of the packaging itself. Developing

active films is a very useful strategy in order to prevent the growth of spoilage microorganisms, hence prolonging the shelf-life of the food products and maintaining their quality. Some of the most widely studied natural bioactive agents are essential oils, phenolic compounds, bacteriocines and enzymes [10, 11].

Lysozyme (LZ) is among the antimicrobial enzymes that have been incorporated into biodegradable polymer materials to obtain active packaging [12]. It is broadly stable, and as it has a high isoelectric point (pI≈11), it is positively charged at the pH of most food products [10]. The antimicrobial activity of this protein is based on its ability to break the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan of the cell walls of Gram-positive bacteria [13]. Egg White lysozyme is considered as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA), and used as a food additive E-1105.

Ethyl lauril arginate (N-α-lauroyl-l-arginine ethyl ester monohydrochloride, LAE), is a cationic surfactant derived from lauric acid, L-arginine, and ethanol. It is considered as one of the most potent food antimicrobial agents with a wide spectrum of antimicrobial activity [12]. It interacts with the cell membranes and causes the membrane protein denaturation, which increases its permeability and causes cell growth inhibition or even death [14].

LAE can be metabolized to yield digestible compounds, which is why it is considered GRAS by FDA, and it has been accepted as food additive E243 [15, 16] It has recently been incorporated into food packaging materials, such as polyethylene terephthalate and polypropylene, and even biodegradable

matrices, such as etilen-vinil-alcohol [17] and chitosan [16]. LAE is predominantly hydrophilic and hence tends to be located in the aqueous phase of food, where the antimicrobial activity takes place. It is chemically stable within the range pH 3 to 7, which includes most food products. It is effective even at lower concentrations than other food preservatives, which makes it a promising additive for biodegradable films [16].

The novelty of this study resides in the possibility of enhancing the functionality and added value of starch-gelatin films by means of the incorporation of lysozyme or LAE in order to obtain active packaging materials for food applications, based on their low cost and food compatibility. In this sense, both, the casting method, which are useful for food coating, and thermoplastic processing, with potential industrial scale-up, have been studied. Thus, the aim of the present work was to obtain active films based on starch-gelatine blends, by incorporating LZ or LAE, using both casting and thermoprocessing methods. The effect of active compounds and processing method on the microstructure, functional properties and antimicrobial power against *Listeria innocua* of the blend films was analysed. Total migration values of the different films into distinct food simulants were also determined.

2. Materials and Methods

2.1. Materials

The following materials were used for film preparation: Corn starch (CS) (Roquette Laisa España, S.A., Valencia, Spain); Bovine gelatin type A (BG) (Sancho de Borja, S.L., Zaragoza, Spain); liofilized lysozyme (LZ) (Fluka

Analytical, Sigma–Aldrich Chemie GmbH, Steinheim, Germany); Ethyl lauroyl arginate (LAE) at 10% w/v in ethanol (Vedeqsa, Lamirsa, Terrassa, Spain). All other chemicals were reagent grade supplied by Panreac Química S.A. (Castellar del Vallés, Barcelona, Spain). Tryptic Soy Broth (TSB), Palcam Agar Base, Agar Bacteriological and Buffered Peptone Water were from (Scharlab, Barcelona, Spain). *Listeria innocua* (CECT 910) was provided by the Colección Española de Cultivos Tipo (CECT, Burjassot, Valencia, Spain). *Micrococcus lysodeikticus* ATCC 4698 was purchased from Sigma, Steinheim, Germany.

2.2. Lysozyme activity

The enzyme activity of lysozyme was determined by a spectrophotometric method as described by previous authors [18], using a spectrophotometer (Helios Zeta UV-VIS, Thermo Fisher Scientific, UK). The absorbance reduction, caused by the lysis of *Micrococcus lysodeikticus* (0.015% w/v), for 5 min, was measured at 450 nm and 25 ºC [18].

The cells were suspended at 0.01% (w/v) in phosphate buffer (66.6 mM, pH 6.24), aiming to achieve an initial absorbance of between 0.6 and 0.7 at 450 nm and 25 ºC. A lysozyme solution (200-400 units/ml) was prepared using the same buffer, and 100 μl of this solution were mixed with 2.5 mL of *M. lysodeikticus* suspension. The absorbance was monitored at 25 ºC for 5 minutes. A mixture of 2.5 mL of *Micrococcus suspension* and 100 μL of buffer was used as blank.

The initial slope of the absorbance *vs.* time curve was used to quantify the enzymatic activity. This was expressed as units of lysozyme per mg (U/mg).

One unit corresponds to a change in absorbance of 0.001 in 1min [19]. Each analysis was carried out in triplicate.

2.3. Film preparation

Three different film forming formulations were prepared using corn starch and bovine gelatin (wt. ratio 1:1) as blend matrix, using glycerol as plasticizer. Control films (CF) and films with lysozyme (LZ) or LAE were considered. Both bioactive compounds were added at a polymer:compound wt.ratio of 1:0.1. Two different techniques were used in order to obtain each film formulation, namely casting (C films) and melt blending plus thermocompression (P films). The amount of plasticizer was fitted in each process to obtain handling films by using the minimum amount as possible to reduce its negative effects. In the case of casting films, glycerol was added at 25 wt.% with respect to the total polymer mass, whereas this ratio was 30 wt.% for thermo-processed films. Therefore, six film types were obtained: control films (CCF and PCF), films with LZ (CLZ and PLZ), and films with LAE (CLAE and PLAE), by using casting or thermocompression, respectively.

To obtain casting films, CS was dispersed in distilled water (2% wt.) and stirred for 5 minutes. In order to induce starch gelatinization, it was immersed in a thermostatic bath at 100ºC for 30 min, and then cooled down to room temperature. BG dispersion (2% wt) was prepared at 40ºC under magnetic stirring for 30min. Additionally, LZ dispersion at 10 wt.% was prepared under stirring at 800rpm for 20min at 25ºC. CS and BG dispersions were mixed in the adequate proportions to obtain the control films (CCF). Glycerol (25g/100 g

polymer) and LZ or LAE (10% w/v in ethanol) solutions were added to obtain CLZ and CLAE films with 10 g of active/100 g polymer. The mass of film forming dispersion corresponding to 1.5 g solids was poured in Teflon plates (150 mm diameter) and dried for 48 h at 45%RH and 25 ºC. Dried films were separated from the plates and conditioned for one week at 25ºC and 53% RH (in desiccators with saturated solutions of magnesium nitrate) prior to analyses.

Compression molded films were obtained by mixing the dry components, CS, BG (and LZ when present) in the proportions defined. The dry blend was mixed with glycerol and water using polymers:glycerol:water mass ratios of 1:0.3:1.1. For films containing LAE, the corresponding amount of the LAE ethanol solution was added to the polymer-glycerol-water blend.

Each mixture was hot-blended in a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 160ºC and 8 rpm for 10 minutes until a homogeneous blend was obtained. The pellets obtained were conditioned for one week at 25ºC and 53%RH using saturated solutions of magnesium nitrate. The films were obtained by compression molding using a hot-plate press (Model LP20, Labtech Engineering, Thailand). Four grams of the blend were preheated at 160ºC for 5 min in the press plate and then pressed at 160ºC and 30 bar for 2 min, followed by 130 bar for 6 min. Thereafter, a cooling cycle to 6ºC for 3 min was applied. The films were finally conditioned in the same way as those obtained by casting.

2.4. Microstructural and physical film characterization

169 2.4.1. Film microstructure

170 Cross section and surface images of the films were obtained by Field Emission
171 Scanning Electron Microscopy (FESEM), with a microscope ZEISS®, model
172 ULTRA 55 (Germany). Prior to analysis, the samples were conditioned in
173 desiccators containing P_2O_5 for 48h. For the cross-section observations,
174 samples were cryofractured by immersion in liquid nitrogen. All the samples
175 were mounted on cupper stubs and coated with platinum.

176

177 2.4.2. Tensile properties

178 The tensile behavior was analyzed following the standard method [20] using a
179 texture analyzer (TA-XTplus, Stable Micro Systems, Surrey, United Kingdom).
180 Twelve replicates per film sample, using film strips (25mm wide, 100mm long),
181 were considered. Prior to every test, the film thickness was measured at four
182 different points by using a hand-held digital micrometer (Electronic Digital
183 Micrometer, Comecta S.A., Barcelona, Spain). Equilibrated film specimens were
184 mounted in the film extension grips and stretched at 50 mm min^{-1} until breaking.
185 Elastic modulus (EM), tensile strength (TS) and elongation at break (% E) were
186 determined from stress–strain curves, estimated from force–deformation data.

187

188 2.4.3. Optical properties: translucency, color and gloss

189 The reflectance spectra of the films (400 to 700nm) were obtained with a
190 spectrocolorimeter MINOLTA, model CM-3600d (Minolta CO, Tokyo, Japan), on
191 both a black (R_0) and a white (R) background of known reflectance. The internal

192 transmittance of the films (T_i) was calculated from these spectra, as an indicator
193 of the film transparency, using the Kubelka–Munk theory [21] for multiple
194 scattering. The reflectance for an infinite film thickness, R_∞, was also determined
195 to obtain color CIE-L*a*b* parameters (CIE, 1986), using illuminant D_{65} and
196 observer 10º. Color coordinates, Lightness (L*) chrome (C_{ab}*) and hue (h_{ab}*), as
197 well as the whiteness index (WI) of the samples, were calculated according
198 previous works [22]. Six samples per formulation were measured.

199 The gloss of the films was measured according to the standard method [23] at 60º
200 incidence angle, using a flat surface gloss meter (Multi.Gloss 268, Minolta,
201 Germany). Six samples per formulation and three measurements per sample
202 were taken. For casting films, measurements were taken on the side which was
203 in contact with air during drying. Results were expressed as gloss units, relative
204 to a highly polished surface of black glass standard with a value near to 100.

205

206 2.4.4. Moisture content and barrier properties

207 The moisture content of film samples previously conditioned at 53% RH was
208 determined in six samples per formulation. Samples were dried in a vacuum
209 oven (60ºC-24h) and, subsequently, conditioned in desiccators with P_2O_5 until
210 constant weight was reached. The results were expressed as g of water per g of
211 dry film.

212 The water vapour permeability (WVP) of the films was determined following a
213 modification of the gravimetric method [24], as described by other authors [25]. Six
214 samples (35mm diameter) per formulation were analyzed. Film thickness was
215 measured with a hand-held digital micrometer (Electronic Digital Micrometer,

Comecta S.A., Barcelona, Spain) at six points. The film samples were secured in Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium) containing 5ml of distilled water (100%RH). Then, the cups were placed in a pre-conditioned cabinet at 25 ºC and 53% RH using magnesium nitrate saturated solutions. In order to reduce the resistance to transport of water vapour, a fan was placed above each cup. The cups were weighed periodically (±0.00001, ME36S Sartorius, Germany) every 1.5 h, until steady state had been reached (24h).

The oxygen permeability (OP) of the films was determined by using an OX-TRAN (Model 2/21 ML Mocon Lippke,Neuwied, Germany) following the standard method [26]. Measurements were carried out at 53% RH and 25ºC on samples with 50cm^2 of exposed area. The sample thickness was measured at six points before the test using a hand-held digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain). Two replicates per formulation were made. The OP was calculated from the oxygen transmission rate, taking into account the film thickness.

2.4.5. Film solubility and swelling in water

Water solubility and swelling of the films were determined by using a modification of the method described by Balaguer et al., (2011) [27]. Film samples of 3x3 cm^2 were initially conditioned for 2 weeks in a desiccator over P_2O_5 (zero theoretical equilibrium moisture content) to obtain dried films. Weighed (W_d^i) dried film samples were immersed in 10 mL of distilled water, gently stirred and kept in contact for 24h at 25ºC. Then, the samples were taken out from the

solvent, gently drained and weighed (W_w^f). The total water content of the wet films was determined by conditioning them in desiccators over P_2O_5 till constant weight (W_d^f). Three replicates per formulation were run. The solubility of the films was expressed as the weight loss (WL%) of the samples (Equation 1) in g/100 g of dried film, while the swelling capacity was estimated as the water uptake (ΔW) (Equation 2) and expressed as g water/100 g of final dried film.

$$WL\% = \frac{W_d^i - W_d^f}{W_d^i} \cdot 100 \qquad \text{(Equation 1)}$$

$$\Delta W\% = \frac{W_w^f - W_d^f}{W_d^f} \cdot 100 \qquad \text{(Equation 2)}$$

2.4.6. Overall Migration

Overall migration tests of the films conditioned for 1 week at 53% RH were carried out following the current legislation [28]. Film samples with a total area of 20.8 cm^2 were immersed in 50 mL of different food simulants: distilled water, simulant A (ethanol 10% v/v, simulating hydrophilic foods), simulant B (acetic acid 3% w/v, simulating low pH hydrophilic foods) and simulant D2 (isooctane, simulating lipophilic foods with free fats at the surface). All the samples were kept in contact with the simulants for 10 days at 20ºC, to simulate any food contact under frozen and refrigerated conditions. After incubation, the film samples were removed from the simulants, which were evaporated to dryness. The final mass of the residues determines the overall migration value. The results were expressed as mg of total constituents released per dm^2 of film. All the tests were run in duplicate.

263

264 2.4.7. Thermogravimetric analysis

265 The thermal stability of the films was analyzed using a thermogravimetric
266 analyzer (TGA/SDTA 851e, Mettler Toledo, Schwerzenbach, Switzerland).
267 Approximately 3 mg of preconditioned sample were used in each test. The
268 sample was heated from room temperature to 600 ºC, under nitrogen flow (50
269 mL/min), at 10ºC/min. Two replicates per formulation were run.

270

271 *2.5. Antimicrobial characterization*

272 The antimicrobial activity of the films with LZ and LAE was analyzed using the
273 Gram positive bacterium *Listeria innocua* (CECT 910). The strain used, initially
274 frozen in TSB with 30% glycerol, was regenerated by inoculation in 10 ml TSB.
275 After incubation (24 h at 37ºC), 10μl were transferred into 10 ml TSB, which
276 was incubated for 24 h at the same temperature to obtain the work culture.

277 Agar plates with 10mL of TSA-NaCl (3%) were inoculated with 10^2 CFU/cm^2 of
278 *L.innocua* (diluted from the work culture), and completely covered by a film with
279 the same surface as the plate. An inoculated plate without film was used as
280 inoculum control, and films without antimicrobial agents (CCF and PCF) were
281 used as control films. All the plates were incubated at 37ºC for 24 h, and
282 bacterium counts were performed at different times of incubation (0, 5 and 24
283 h), using a specific medium for *Listeria*, Palcam agar. All the tests were run in
284 duplicate.

285

2.6. Statistical analysis

The statistical analysis of the data was performed through analysis of variance (ANOVA) using Statgraphics Centurion XVI s for Windows 5.1 (Manugistics Corp., Rockville, Md.)

3. Results and Discussion

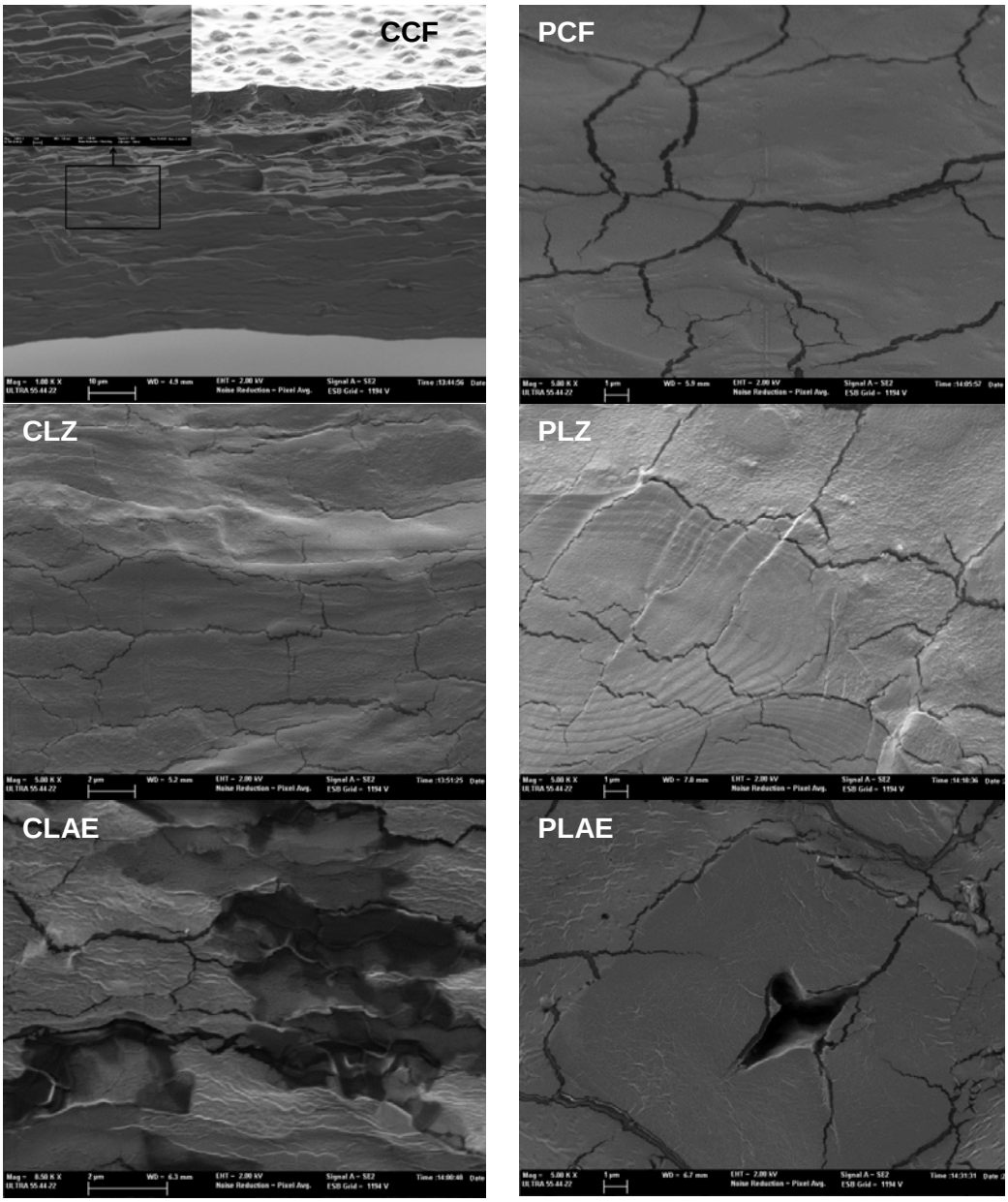
3.1. Microstructural and physical film characterization

3.1.1.Microstructure of the films

Figures 1 and 2 show the images of the cross sections and surface of the films, obtained by FESEM. Figure 1 illustrates the differing internal microstructure of the control films (starch and gelatin blends) obtained by casting and compression molding. The former (CCF) shows a stratified arrangement of the polymers, caused by their limited miscibility, which forms during the drying step. Phase separation occurs during this step and the gelatin-rich phase (with low density) is predominant at the top of the film, whereas the starch-rich phase (with higher density) remains mainly distributed at the bottom of the film. This polymer arrangement is also evidenced at surface level (Figure 2), where the gelatin domains can be observed as globular formations, as previously seen in casting films of similar composition by other authors [6]. Polymer phase separation could also be observed in control films obtained by compression molding (PCF), even though no gravitational stratification can take place in this

309 case. Figure 1 shows the lack of complete adhesion of both phases at the
310 interface of different polymer domains. The PCF surface (Figure 2) shows a
311 fibrous structure which could be attributed to the gelatin chain aggregations.
312 This polymer tends to acquire a tridimensional structure similar to collagen,
313 through the aggregation of its helical conformation [6].

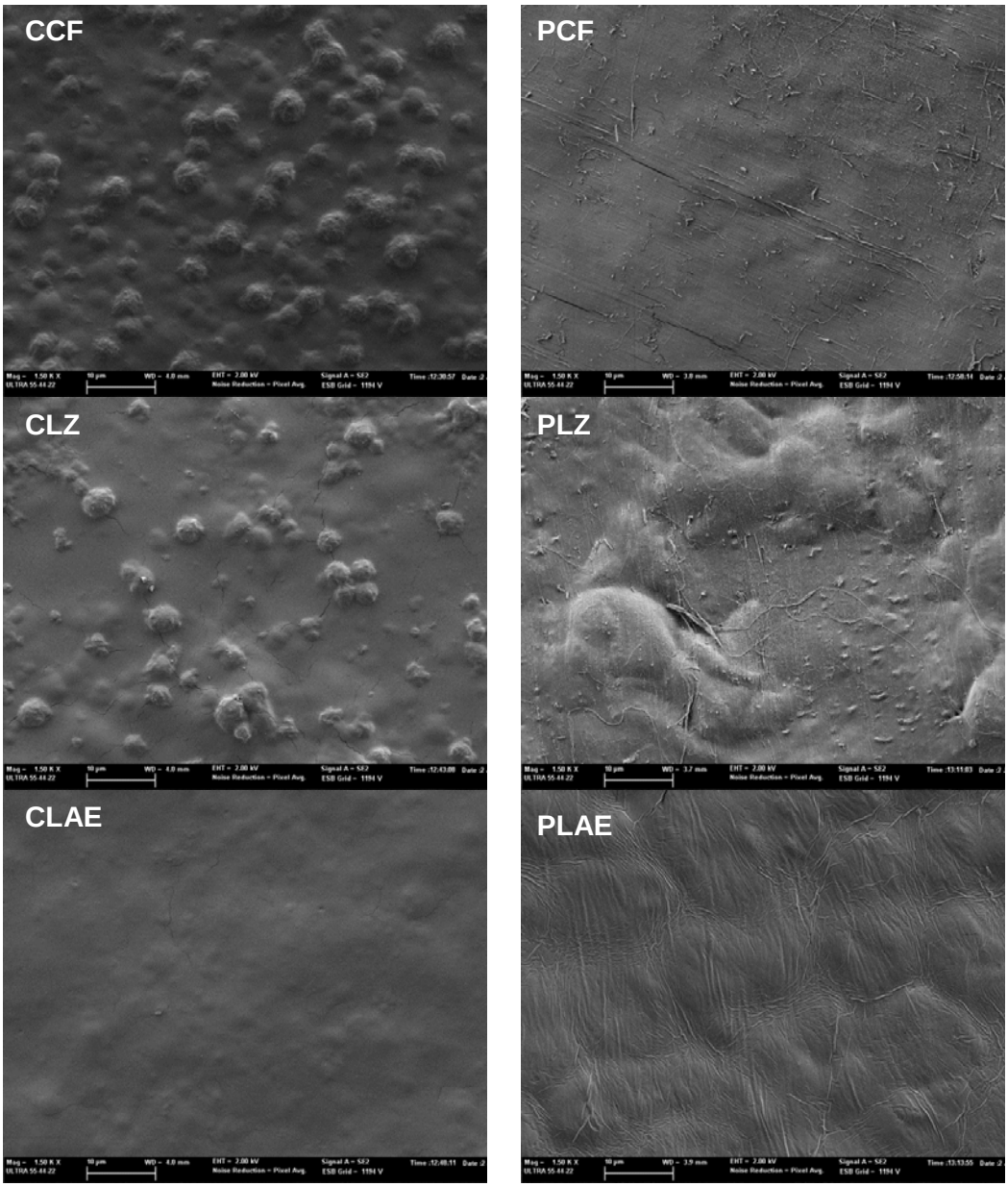
314 The incorporation of LZ into the films seems to enhance the interactions
315 between the two polymers, hence favouring the blending, both for casting and
316 compression-molding films. Figure 1 shows some degree of polymer phase
317 separation in CLZ and PLZ films, but the interfacial adhesion appears to be
318 improved as compared to the control films. The surface of casting films with
319 lysozyme (Figure 2) also exhibits globular forms, but to a lesser extent than the
320 control films. PLZ samples show less clearly delineated fibrous structures than
321 PCF control films, thus indicating a better degree of gelatin and starch phase
322 blending.



323

324 **Figure 1.** FESEM images of the cross section of control films and films
325 containing LZ and LAE, obtained by casting (left) and compression molding
326 (right). Bar in CCF corresponds to 10 μm and for the included micrograph, at
327 higher magnification, to 2 μm; bars in CLZ and CLAE correspond to 2 μm and,
328 in PCF, PLZ and PLAE, to 1μm.

329



330

331 **Figure 2.** FESEM images of the surface of control films and films containing LZ
332 and LAE, obtained by casting (left) and compression molding (right). Bars in all
333 micrographs correspond to 10μm.

334 In films with LAE, the starch and gelatin domains were smaller, which suggests
335 the improvement of the polymer blending capacity caused by this surfactant
336 compound. The internal structure of PLAE films showed some gaps, probably
337 caused by air incorporation during the homogenization step, favoured by the

surfactant effect of LAE. The surface of films with LAE was more homogeneous, coherently with the better polymer homogenization due to the interfacial action of this compound.

So, the amphiphilic nature of LZ, and especially of LAE, contributed to enhance blending capacity of starch and gelatin phases, thus giving rise to a different microstructural arrangement in the polymer matrix. Likewise, casting or compression molding processes seriously affected film microstructure due to the differences in the polymer chain interactions which occurred in the aqueous solution during film drying or in the melt blending process, as well as the gravitationally induced stratification of separate polymer phases during the drying step of casting films.

3.1.2. Tensile properties

Table 1 shows the film thickness values, along with the tensile parameters: elastic modulus (EM), tensile strength at break (TS) and percentage elongation at break (%E) of the films. The compression molding method led to a significantlly increased film thickness, as compared to the casting method, due to the low flowability of the material in the compression step.

The tensile parameters of the casting control films (CCF) were in the order of those reported by previous works [6] for films of similar composition. In general, thermo-pressed films were less rigid, less resistant to fracture and more stretchable than casting films, which can be explained by their different microstructure. In the casting films, both polymer phases are partially separated in the film forming solution, but the polymer chains can extend and interact to a

different extent throughout the drying step, thus giving rise to two independent networks with strong chain attraction within each homopolymer phase and weaker interactions at the polymer interfaces. On the other hand, in the films obtained by compression molding, polymer domains of different sizes were produced during the melt blending step, which offers less opportunity for the development of molecular interactions. A great contact surface between the two polymer phases is produced, with weak adhesion forces at the interface. Differences in the film thickness for casting and thermo-processed films will also affect their tensile properties.

Lysozyme addition affected the tensile behavior of the compression molded films. As compared to the corresponding control film (PCF), lysozyme addition did not significantly affect the tensile properties of the thermoprocessed films, although the interfacial action of LZ in thermo-compressed films resulted in an improved polymer adhesion (Figure 1). However, casting films with lysozyme (CLZ) were significantly less rigid and resistant, but more stretchable than the corresponding control film (CCF).

On the other hand, LAE addition affected the tensile behavior of the films obtained by both methods. In comparison with control films, films with LAE became less rigid and resistant ($p<0.05$) in both casting and thermo-compression methods. This reduction of the strength parameters is justified by the improved dispersion of starch and gelatin phases (smaller domains) caused by LAE addition, thus exhibiting greater interfacial area where adhesion forces are weaker. The film extensibility was increased in casting films (CLAE), and notably reduced for those obtained by compression molding (PLAE). The increased extensibility of casting films could be related to the sliding of the

polymer chains during the tensile test favoured by the interfacial action of LAE, whereas in the control film (CCF) the intermingled phases show more resistance and lower deformability. On the contrary, the interfacial action of LAE seems to be less effective in the thermo-compression process, since polymer phases are structured as adhered smaller domains (greater interfacial area) with some gaps, which makes the film rupture easier, as compared to the corresponding control film (PCF).

On the other hand, the degree of crystallinity in both polymer phases could also have a remarkable effect on the tensile response of the films obtained by the different methods. In the casting films, aggregation and crystallization phenomena are liable to occur during the drying step due to the high molecular mobility of the polymer chains, whereas this molecular arrangement is much less probable in thermo-compressed films, which will exhibit more amorphous and less resistant structures [29].

3.1.3. Optical properties

The values of the optical parameters of the films (lightness, chrome, hue, whiteness index and gloss at 60º) are reported in Table 2. The film processing method significantly affected the colour of the films. Compression molding yielded darker films with higher chrome and lower hue than those obtained by casting ($p<0.05$). The films obtained by compression molding had some reddish coloration, indicating that some browning reactions take place during the thermal process. On the contrary, the casting films were almost white (with low chrome, regardless of the hue value).

411 Lysozyme addition did not affect the colour of casting films, whereas in
412 compression molded films, this compound led to a lightness and hue reduction,
413 and a chrome increase ($p<0.05$), giving rise to more brownish films, as
414 compared to PCF. LAE incorporation in casting films resulted in a significant
415 decrease in lightness and chrome, whereas hue increased ($p<0.05$). However,
416 when this compound was incorporated into compression molded films, chrome
417 was significantly reduced ($p<0.05$) while WI increased with respect to the
418 corresponding control films, thus indicating that less film browning was
419 produced in this case. In fact, of the compression molded films, PLAE were the
420 least affected by browning. The whiteness index values were coherent with the
421 colour parameters, and they were significantly higher in casting films ($p<0.05$).
422 These results suggest that melt blending and compression molding could not
423 represent a convenient strategy to obtain starch-gelatin films since the dark
424 brown discoloration evidences some degradation of the materials occurring
425 during the thermal process, although LAE incorporation mitigated these effects.

426 For both processing methods, LZ and LAE addition resulted in a significant
427 gloss increase ($p<0.05$) and no significant effect of the method was observed.
428 The gloss increase can be attributed to the surface roughness reduction (fewer
429 globular formations), as deduced from the FESEM observations in Figure 2.

430 Figure 3 shows the internal transmittance (T_i) spectra of the films, as an
431 indicator of their transparency. The processing method was the factor that
432 mainly affected this property; the films obtained by casting were more
433 transparent (higher T_i values) than those obtained by compression molding. The
434 addition of LZ and LAE only affected the transparency of the compression
435 molded films. With respect to the control film (PCF), PLZ showed the lowest T_i

values, hence being the least transparent films, whereas LAE addition led to a transparency increase in compression molded films (PLAE).

The reduction of T_i at low wavelength may be attributable to the browning products absorbing light between 400 and 500nm. These compounds may result from Maillard reactions (condensation reactions between protein amine groups and carbohydrate carbonyl groups) or the caramelization of the carbohydrates, both enabled by the high process temperature. In this sense, it is remarkable that LZ and LAE provoked different effects, both having amino groups but probably with different reactivity.

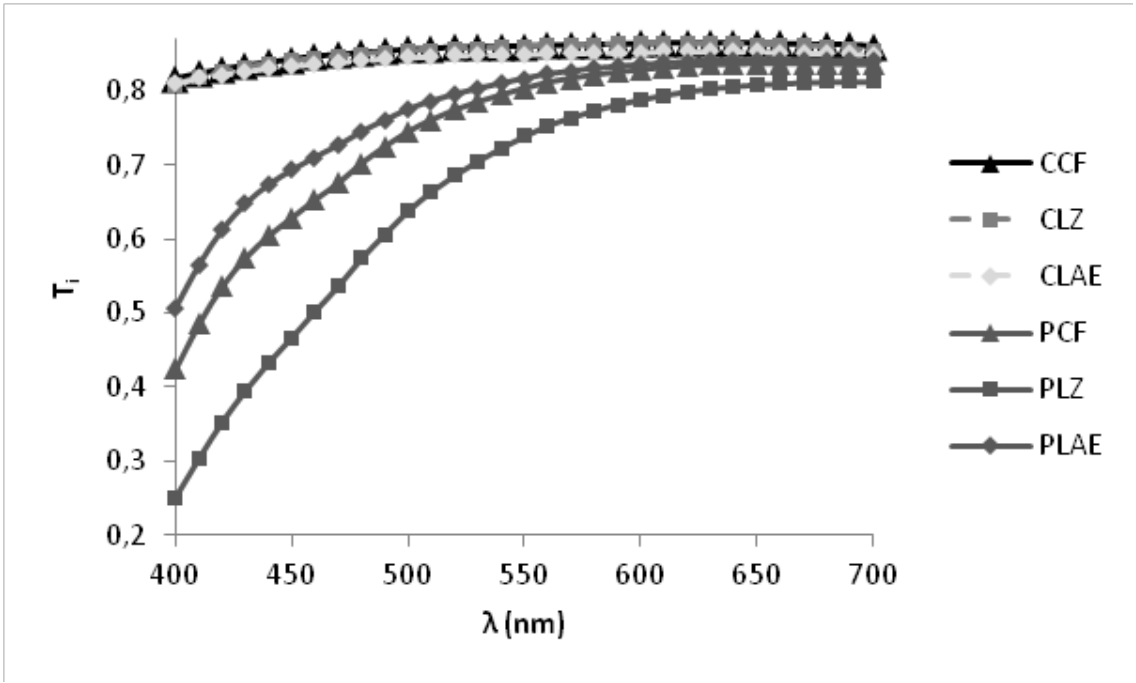


Figure 3. Spectral distribution of internal transmittance between 400 and 700 nm, for the different film formulations obtained by casting (C) and compression molding (P).

3.1.4. Moisture content and barrier properties

Table 3 shows the equilibrium moisture content of the films (X_w, g water per g dry film), their water vapor permeability (WVP) and their oxygen permeability (OP). The moisture content was slightly lower in thermoprocessed films and casting films with LAE, whereas LZ casting films showed the highest value, although the differences are not very remarkable and could be attributed to the different structural arrangement of polymers which could imply a different ratio of active points for water adsorption. The reduction of water affinity provoked by the LAE addition in the films, suggests that a slightly more hydrophobic matrix is produced in this case, whereas the opposite trend was observed for LZ films. Nevertheless, these effects disappeared in thermoprocessed films, where browning reactions, occurred during heating, may involve polar groups, thus contributing to the overall reduction of the number of active sites for water adsorption.

The barrier properties (WVP and OP) of the casting control films were similar to those previously reported for starch-gelatin casting films [6]. However, compression molded films exhibited significantly higher values of WVP and OP ($p<0.05$) than casting films. For both film forming techniques, the incorporation of LZ reduced OP (6, 20%, for C and P films) and increased WVP (19, 17%, for C and P films). Contrarily, LAE significantly increased OP (65, 279% for C and P films) and reduced WVP (28, 30%, for C and P films) for both film types. This different response could be explained by the fact that the different structural properties and the induced changes in the hydrophilic character of the matrix (different water adsorption capacity) give rise to different transport rates for water and oxygen in the film. In this sense, compression molded films, exhibiting multiphasic structures with weak interphase adhesion, and being less

476 compact than casting films, allowed for a greater degree of mass transport than
477 casting films. The effect the processing method had on mechanical and barrier
478 properties coincides with that previously reported by other authors for gelatin-
479 starch films [9].

480 The opposite effect of LZ and LAE on WVP and OP agrees with the previously
481 mentioned reverse effect of these compounds on the water affinity of the films;
482 whereas LZ enhanced the matrix hydrophilic character, LAE promoted its
483 hydrophobicity. Consequently, water solubility in the matrix increases in LZ
484 films, whereas oxygen solubility increases in LAE films, thus affecting the
485 permeation behavior of each molecule in the matrix in an opposite way.
486 Likewise, according to the multiphasic structure of the films, tortuosity factor for
487 mass transport of permeants will also affect the mass transport rate [30].
488 Moreover, the obtained values for water vapour barrier properties of starch-
489 gelatin blends were not in the established range of water vapour transmission
490 rate for food systems' requirements [31] and reduction would be necessary, which
491 could be also obtained by cross linking strategies. Nevertheless, oxygen barrier
492 properties of these films cover the whole range of food packaging requirements
493 [31].

494

495 *3.1.5.* Film water solubility and swelling and migration capacity

496 Figure 4 shows the values obtained for the water solubility and swelling capacity
497 of the different samples, expressed as weight loss (%) and water uptake (%),
498 respectively. Control films obtained by both casting and thermo-pressing
499 methods (CCF and PCF) showed a similar water solubility to that reported by

Fakhouri et al. (2013). Neither film composition nor the processing method led to significant changes in the film solubility. On the contrary, films obtained by casting exhibited a significantly greater water uptake ($p<0.05$) (Figure 5b). In all likelihood, the chain packing stemming from the drying of the film forming solutions is able to partially recover the hydrated form, binding a high water ratio, whereas the different polymer chain packing obtained from melt blending has a much lower water binding capacity in line with the development of different chain interactions at high temperatures without water. In addition, the loss of hydroxyl groups occurred during the caramelization process in compression molded films, could contribute to reduce the water uptake values. Both LZ (more markedly) and LAE enhanced the water uptake capacity of the casting films, whereas no significant effect was observed in compression molded films.

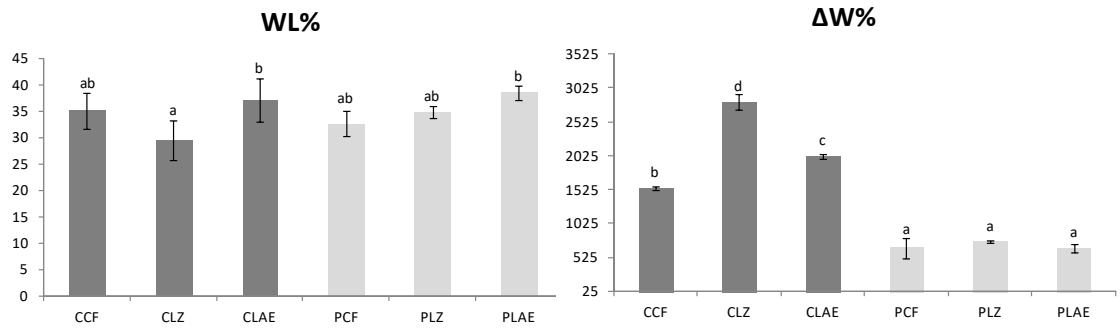


Figure 4. a) Weight loss of dried films (WL%, g/100 g dried film) after 24h of immersion in distilled water at 25ºC. Water uptake (ΔW%, g water/100 dried film) of the films after 24h of immersion in distilled water at 25ºC. Average values and standard deviation. Different letters (a,b,c,d) indicate significant differences among the different formulations.

519 Related with the solubility of the films in water systems such as foods, Table 4
520 shows the overall migration values obtained for the different films in water and
521 some food simulants. For all the films, migration values in hydrophilic simulants
522 (water, 10 % ethanol and 3 % acetic acid), exceeded the established limit for
523 the overall migration of 10 mg/dm^2 by the current law. In the most hydrophobic
524 simulant (isooctane), films obtained by compression molding showed
525 significantly lower overall migration values than those obtained by casting,
526 meeting the established limit. When 10 % ethanol was used, a different
527 behavior was observed for casting and thermoprocessed films; casting films
528 exhibited the lowest migration levels among all the hydrophilic simulants, these
529 being closer to those obtained in isooctane, whereas thermoprocessed films
530 showed the highest values. In general, the film processing mainly affected the
531 the overall migration behavior of the films, while the addition of LZ or LAE
532 slightly promoted the migration of film components in polar simulants. On the
533 basis of these results, different strategies, such as a crosslinking process,
534 would be required in order to reduce the migration capacity of the starch-gelatin
535 films in aqueous food systems.

536

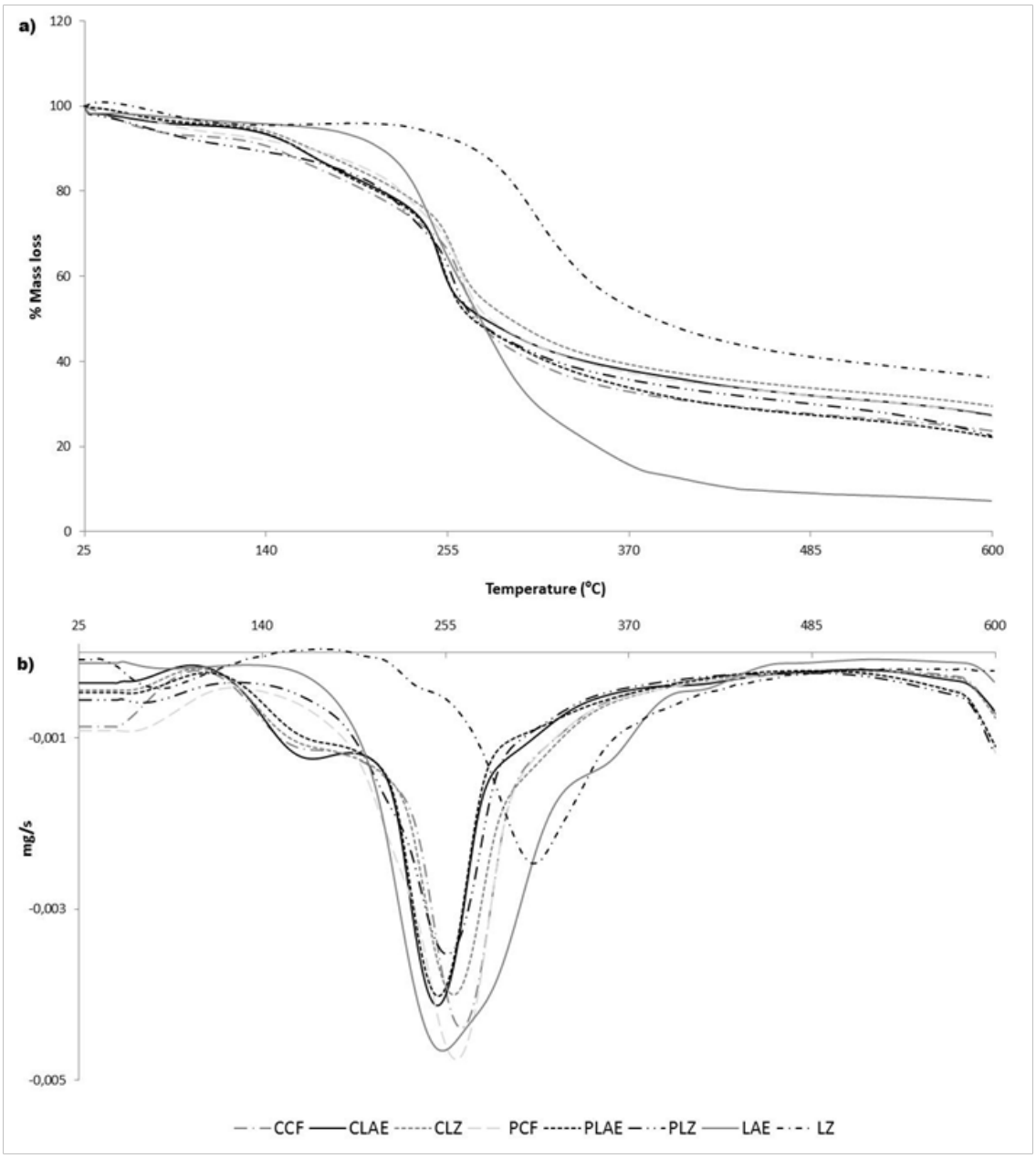
537 *3.1.6.* Thermogravimetric analysis

538 Figure 5 shows the obtained thermograms for the different films and pure LZ
539 and LAE. Likewise, in Table 5, the values of the initial degradation temperature
540 (T_0) and the temperature at the maximum degradation rate (T_{max}) for the
541 different films and the pure active compounds are summarized. These results
542 allow for the elucidation of the thermal stability of the material as a function of

543 the composition, as well as the possible effect of thermal processing, as
544 compared to casting films. The initial degradation temperatures of LZ and LAE
545 were 217ºC and 175ºC respectively. These values are higher than the process
546 temperature (160ºC), which offers some guarantees about their stability during
547 the melt blending process.

548 Nevertheless, all the films obtained by compression molding, except PLAE,
549 showed a T_0 value of around 160ºC, whereas this was about 120ºC for the rest
550 of the films. This indicates that during the thermoprocessing at 160ºC, some
551 degradation compounds were formed from 120ºC onwards, which may be
552 responsible for the coloration of these films, as previously mentioned. The
553 increase of T_0 for the films obtained by thermocompression indicates the
554 different chemical composition attained by the materials after being partially
555 degraded during the thermal process. It is remarkable that the
556 thermocompressed films with LAE (PLAE), did not present significant browning,
557 which is coherent with the lower T_0 value (121ºC) observed in the thermogram.
558 This seems to indicate that there is some thermoprotective effect of this
559 compound in the polymers during the melt blending process. The maximum
560 degradation rate temperature of all the films was between 250ºC and 260ºC; it
561 was slightly lower both in films with bioactive compounds and, except for LAE
562 formulation, in compression molded films.

563 On the basis of these results and the optical analysis, thermal processing of
564 starch-gelatin blends could not represent a good strategy to obtain films due to
565 the occurrence of some degradation reactions, which give rise to dark brown
566 discoloration of the films. Nevertheless, LAE incorporation implied a certain
567 protection against these browning reactions.



568

569 **Figure 5.** Thermogravimetric curves (a) and the first derivative of TGA curves
570 (b) of the film samples and pure antimicrobial compounds (LZ and LAE).

571

572 *3.2. Antilisterial properties of the films and Lysozyme activity*

573 Table 6 shows the results obtained from the analysis of the films'
574 antimicrobial activity. The films with LAE (both CLAE and PLAE) had an
575 absolute bactericidal effect at every control time. On the other hand, none of the

formulations with LZ exhibited antilisterial activity, in spite of its notable enzyme activity (21.803 ± 2.378 U/mg), determined through the absorbance reduction caused by the lysis of *Micrococcus lysodeikticus*[19]. This could be attributed to the immobilization of LZ in the starch-gelatin matrix, which could limit its diffusion from the film surface, taking into account its high molecular weight (14.4 kDa). Therefore, its potential antimicrobial activity would be inhibited.

4. CONCLUSIONS

Whereas the incorporation of LAE to starch-gelatin blend films was very effective at imparting active (antilisterial) properties to the material, LZ did not confer notable antibacterial activity on the films. LAE also improved the interfacial adhesion of the polymers in both casting and thermoprocessing methods, making the blending process easier. The incorporation of LAE notably reduced stiffness and resistance to break in casting films, making them more extensible, which was only observed for LZ in the case of the casting method. Likewise, LAE promoted water vapor barrier properties and increased the oxygen permeability of the films, although the latter was in the range of food packaging requirements. Likewise, this antimicrobial compound enhanced the thermal stability of the blends, which is highly positive for thermoplastic processing at industrial level. Therefore, as it is a promising compound when formulating biodegradable active films obtained by either casting or thermal processing, its incorporation into starch-gelatin films greatly enhanced their functionality and added value. Nevertheless, due to the fact that there is a high degree of overall migration of the films to hydrophilic food simulants, different

strategies, such as the crosslinking process, would be required to limit this aspect.

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689

690 **Table 1.** Values of thickness (mm) and tensile parameters (EM (MPa), TS
691 (MPa) and %E) of the films equilibrated at 53% of RH at 25ºC. Average values
692 and standard deviation.

Formulation	Thickness (mm)	EM (MPa)	TS (MPa)	%E
CCF	0.065 ± 0.006^{a}	1021 ± 194^{d}	33 ± 6^{e}	14 ± 5^{a}
CLZ	0.065 ± 0.004^{a}	461 ± 15^{c}	18 ± 2^{c}	32 ± 9^{b}
CLAE	0.061 ± 0.003^{a}	512 ± 95^{c}	24 ± 7^{d}	35 ± 2^{b}
PCF	0.180 ± 0.014^{b}	110 ± 42^{b}	15 ± 2^{b}	94 ± 19^{c}
PLZ	0.227 ± 0.012^{c}	119 ± 34^{b}	16.4 ± 0.2^{bc}	100 ± 28^{c}
PLAE	0.176 ± 0.013^{b}	21.6 ± 1.8^{a}	3.4 ± 0.3^{a}	29 ± 3^{b}

693 Different letters (a, b, c, d, e) in the same column indicate significant differences among the different
694 formulations ($p<0.05$).

695

696 **Table 2.** Values of the colour parameters (L*, Lightness; C_{ab}*, Chrome; h*, hue),
697 Whiteness Index (WI) and Gloss at 60° for the different film formulations.
698 Average values and standard deviation.

Formulation	L^*	C_{ab}^*	h_{ab}^*	WI	Gloss (60º)
CCF	80.6 ± 0.5^{d}	7.3 ± 0.2^{b}	98.0 ± 0.3^{d}	79.4 ± 0.4^{e}	12.1 ± 0.7^{a}
CLZ	81.4 ± 0.3^{e}	7.4 ± 0.4^{b}	98.6 ± 0.8^{d}	80.0 ± 0.3^{e}	14.1 ± 0.9^{b}
CLAE	73.8 ± 0.6^{c}	4.36 ± 0.13^{a}	104.0 ± 0.9^{e}	73.4 ± 0.6^{d}	18.7 ± 1.3^{c}
PCF	62.32 ± 1.05^{b}	30.4 ± 1.3^{d}	83.6 ± 0.7^{b}	51.6 ± 0.9^{b}	12 ± 4^{a}
PLZ	54.7 ± 0.2^{a}	33 ± 3^{e}	76.5 ± 2^{a}	43.9 ± 1.9^{a}	19 ± 4^{c}
PLAE	62.3 ± 0.7^{b}	26.1 ± 1.8^{c}	85.5 ± 0.4^{c}	54.3 ± 0.2^{c}	19 ± 4^{c}

699 Different letters (a, b, c, d, e) in the same column indicate significant differences among the different
700 formulations ($p<0.05$).

Table 3. Values of the barrier properties (WVP, Water Vapour Permeability; OP, Oxygen Permeability) and equilibrium moisture content (Xw). Average values and standard deviation.

Formulation	WVP (g/Pa·s·m)·10^7	OP (cm^3/m^2·día)·10^{13}	X_w (g water/g dry film)
CCF	6.9 ± 0.2^{b}	1.26 ± 0.03^{a}	12 ± 3^{b}
CLZ	8.2 ± 0.5^{c}	$1,19 \pm 0.02^{a}$	14.0 ± 0.2^{c}
CLAE	5.0 ± 0.6^{a}	2.08 ± 0.07^{ab}	10.3 ± 0.5^{a}
PCF	15.1 ± 1.3^{d}	2.51 ± 0.02^{b}	10.3 ± 0.6^{a}
PLZ	17.6 ± 0.9^{e}	1.99 ± 0.16^{ab}	9.3 ± 0.9^{a}
PLAE	8.1 ± 1.2^{c}	9.47 ± 1.18^{c}	9.7 ± 0.5^{a}

Different letters (a, b, c, d, e) in the same column indicate significant differences among the different formulations ($p<0.05$).

Table 4. Overall migration values (mg/dm^2) of the different films (for 10 days at 20ºC) into different food simulants, distilled water, ethanol (10% v/v) (simulant A), acetic acid (3% w/v) (simulant B) and isooctane (simulant D2). Average values and standard deviation.

Different letters (a, b, c, d) in the same column indicate significant differences among the different formulations for the same stimulant ($p<0.05$).

Different letters (x, y, z) in the same row indicate significant differences among the different simulants for the same formulation

Formulation	Water	Ethanol (10% v/v)	Acetic acid (3% w/v)	Isooctane
CCF	$318 \pm 40^{a,y}$	$108 \pm 15^{a,x}$	$362 \pm 36^{a,y}$	$47 \pm 24^{b,x}$
CLZ	$394 \pm 84^{ab,y}$	$212 \pm 46^{ab,x}$	$673 \pm 35^{bc,z}$	$79 \pm 8^{b,x}$
CLAE	$394 \pm 129^{ab,xy}$	$348 \pm 52^{b,xy}$	$622 \pm 148^{bc,y}$	$126 \pm 99^{b,x}$
PCF	$598 \pm 14^{bc,y}$	$713 \pm 16^{c,z}$	$531 \pm 59^{ab,y}$	$3 \pm 2^{a,x}$
PLZ	$793 \pm 29^{c,yz}$	$853 \pm 7^{cd,z}$	$774 \pm 42^{c,y}$	$3 \pm 2^{a,x}$
PLAE	$735 \pm 164^{c,y}$	$989 \pm 125^{d,y}$	$982 \pm 51^{d,y}$	$2 \pm 2^{a,x}$

716 (p<0.05).

717

718

719 **Table 5.** Onset (To) and peak (Tmax) (maximum degradation rate)
720 temperatures for polymer degradation in the different films.

Formulation	$T_{máx}$ (ºC)	T_0 (ºC)
CCF	264.75 ± 0.11^{e}	119 ± 4^{a}
CLZ	260.09 ±0.12^{d}	124.8 ± 0.7^{a}
CLAE	249.9 ± 0.4^{a}	123.5 ± 1.7^{a}
PCF	261.75 ± 0.11^{d}	162.2 ± 1.4^{b}
PLZ	255.92 ± 0.12^{c}	161.3 ± 1.4^{b}
PLAE	251.3 ± 1.3^{a}	121.6 ± 0.3^{a}
LAE	253. 8 ± 1.1^{b}	175 ± 4^{c}
LZ	310.7 ± 1.6^{f}	217 ± 7^{d}

721 Different letters (a, b, c, d, e, f) in the same column indicate significant differences among the different
722 formulations (p<0.05).

723

724

725

726

727

728 **Table 6.** Effect of the films on the growth and survival of *Listeria innocua* (CECT
729 910) at 37ºC. Bacterial counts obtained at initial time (0 h), 5 and 24 h of
730 incubation. Average values and standard deviation.

	0h	5h	24h
Formulation	**Log (CFU/cm^2)**	**Log (CFU/cm^2)**	**Log (CFU/cm^2)**
Ci**	2.08 ± 0.07^{a,x}	3.91 ± 0.05^{b,y}	7.35 ± 0.02^{b,z}
CCF	2.14 ± 0.04^{b,x}	4.19 ± 0.03^{c,y}	7.40 ± 0.05^{b,z}
CLZ	2.13 ± 0.03^{ab,x}	4.18 ± 0.03^{c,y}	7.40 ± 0.03^{b,z}
CLAE	NDG*	NDG*	NDG*

PCF	$2.25 \pm 0.02^{c,x}$	$3.86 \pm 0.08^{b,y}$	$7.35 \pm 0.04^{b,z}$
PLZ	$2.13 \pm 0.02^{ab,x}$	$3.77 \pm 0.06^{a,y}$	$7.24 \pm 0.07^{a,z}$
PLAE	NDG*	NDG*	NDG*

Different letters (a, b, c, d, e) in the same column indicate significant differences among the different formulations ($p<0.05$).

Different letters (x, y, z) in the same row indicate significant differences among the different times of incubation for the same formulation ($p<0.05$).

* No Detected Growth.

** Inoculum Control.