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Antimicrobial packaging based on a LAE containing zein coating to control foodborne pathogens in chicken soup

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ABSTRACT

In this study, zein coatings containing Lauroyl-L-arginine ethyl ester monohydrochloride (LAE) were developed to be applied on polypropylene films and manufacture an active food packaging. The concentration of LAE and the addition of a suitable plasticizer (glycerol or oleic acid (OA)) were the main variables considered. Active plasticized zein films, with glycerol or oleic acid were characterized in terms of release kinetics, mechanical, barrier, optical, and antimicrobial properties. Results showed that active agent concentration, (5 and 10%), had no-significant effect on mechanical and WVP properties of the plasticized films. Films plasticized with OA presented greater water resistance, UV-light opacity, and water barrier properties than glycerol-plasticized films. On the contrary, the latter had better antimicrobial properties. The analysis of LAE release kinetics from films to different food simulants revealed different behaviours, depending on both film formulation and food simulant. Despite the lower water resistance of coatings containing glycerol, bags based on polypropylene/glycerol plasticized zein containing 10% of LAE presented a great antimicrobial activity in tests with chicken soup (real food system) contaminated with pathogen bacteria, concretely, the films showed 3.21 Log reduction against *Listeria monocytogenes* and 3.07 log reductions against *Escherichia coli*. These results suggest a promising strategy on the use of LAE-containing zein in active food packaging to control foodborne pathogens.

1. Introduction

Nowadays, multifunctional packaging systems containing active substances have been designed to answer the safety concerns of industrial products as well as customers' demands for using recyclable and biodegradable materials in food packaging. The worldwide emergence of antibiotic resistance against foodborne pathogens resulted in challenges in food security (Chouhan et al., 2017). In this regard, using essential oils from aromatic and medicinal plants as antimicrobial agents in food packaging is a blooming trend to combat resistant bacteria (Abbaszadeh et al., 2014; Kashiri et al., 2016). However, essential oils are volatile and unstable compounds and thereby, easily evaporate under environmental condition reducing their antimicrobial efficiency. Moreover, essential oils in food packaging might change the flavour and/or odour of food products. Therefore, many researchers are seeking for the ideal antimicrobial agent, which should be flavourless, resistant to high temperatures, and colourless. Recently, lauric arginate (Lauroyl-L-arginine ethyl ester monohydrochloride, LAE) has been revealed as a promising antimicrobial agent against different foodborne pathogen (Bakal and Diaz, 2005; Bonnaud et al., 2010; Kang et al., 2014; Kashiri et al., 2016; Rodriguez, 2004; Ruckman et al., 2004; Theinsathid et al., 2012). In addition, LAE has been incorporated in different polymers matrix including ethylene-vinyl alcohol copolymer (EVOH) (Muriel-Galet et al., 2012), chitosan (Higueras et al., 2013b), or zein (Kashiri et al., 2016) to enhance films functionality.

Zein, a fraction of endosperm tissue of corn and a major by-product

Abbreviations: a*, Colour coordinate indicating red/green; b*, Colour coordinate indicating yellow/blue; CECT, Spanish Type Culture Collection; CFU, Colony forming units; D, diffusion coefficient; EB, elongation at break; EVOH, ethylene-vinyl alcohol copolymer; HPLC, High performance liquid chromatography; K, partition coefficient; L, Thickness; L*, Colour coordinate indicating lightness; LAE, Lauroyl-L-arginine ethyl ester monohydrochloride; LRV, log reduction value; LSD, Least significant difference; OA, Oleic acid; PLA, polylactic acid; PP, Polypropylene; RH, Relative humidity; SEM, Scanning electron microscopy; TS, Tensile strength; UV, Ultraviolet; v/v, Volume to volume; V_P, volume of polymer; V_S, volume of simulant; w/w, Weight to weight; WS, Water solubility; WVP, water vapour permeability; YM, Young's modulus; ZEO, *Zataria multiflora Boiss.* essential oil; ZG, Zein film plasticized with glycerol; ZG-10%LAE, Zein film plasticized with glycerol containing 10% of LAE; ZG-5%LAE, Zein film plasticized with glycerol containing 5% of LAE; ZO, Zein film plasticized with oleic acid; ZO-10%LAE, Zein film plasticized with oleic acid containing 10% of LAE; ZO-5%LAE, Zein film plasticized with oleic acid containing 5% of LAE; ΔE, Colour difference

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of the bio-ethanol industry, is considered a valuable candidate for packaging applications due to its bio-degradability, sustainability, good film-forming ability and adhesive/cohesive properties (Biswas et al., 2009; Singh et al., 2010). Moreover, zein-based films are considered excellent biomaterial vehicles for antimicrobial agent delivery in food packaging applications (Kashiri et al., 2016).

Achieving an adequate controlled release of agents in active packaging is critical to keep active substance concentration above the minimum inhibitory concentration against pathogenic or spoilage microorganisms during product shelf-life (Franssen et al., 2004). From this point of view, different strategies have been suggested based on: multilayer films (Buonocore et al., 2004; Lopez-Rubio et al., 2006), smart bio-polymer blending (LaCoste et al., 2005), matrix cross-linking (Buonocore et al., 2003), encapsulation technology (Parris et al., 2005; Silva-Weiss et al., 2018; Tampau et al., 2018), and incorporation of various type of fatty acids (Arcan and Yemenicioğlu, 2014). Fatty acids have been also incorporated in zein films as plasticizers to overcome brittleness, poor process ability, low elongation, and to improve water barrier properties and visual appearance (Padua and Wang, 2002). Depending on the composition and structure of film matrix (Loeffler et al., 2014), the addition of oleic acid into a complex active system might create molecular interactions which modify film antimicrobial properties (Weiss et al., 2015).

From this point of view, the interactions of LAE in film forming solutions due to its reactions with diverse functional groups are a big challenge. Indeed, such interactions can be used as a strategy for the controlled release of LAE in bio-packaging. Therefore, the aims of this work were: (1) to evaluate the impact of LAE concentration on physical-mechanical properties of plasticized zein films, (2) to evaluate the effect of plasticizers in active zein films to develop suitable antimicrobial films for the preparation of active bilayer packaging materials, and (3) to investigate the antimicrobial efficiency of active zein based films against *Listeria monocytogenes* and *E. coli* as microbiological model systems, in vitro and in chicken soup (a real heterogeneous system).

2. Materials and methods

2.1. Materials

Deodorized Kobayashi zein powder was purchased from CBC-Iberia (Barcelona). Ethyl-Nα-dodecanoyl-L-arginate hydrochloride ($C_{20}H_{41}N_4O_3Cl$) was kindly provided by Vedeqsa Grupo LAMIRSA (Terrassa, Barcelona, Spain). Oleic acid, trifluoroacetic acid and glycerol were obtained from Sigma (Madrid, Spain). Ethanol, acetic acid, and acetonitrile were purchased from Scharlau (Barcelona, Spain), deionized water was supplied by a Millipore Milli-Q Plus purification system (Molsheim, France).

2.2. Bacterial strains

Foodborne microbial strains, Gram-positive bacteria *Listeria monocytogenes* CECT 934 (ATCC 19114) and Gram-negative bacteria *Escherichia coli* CECT 434 (ATCC 25922), were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain) and selected for using in the assays because of their relevance in food infections. Culture medium was purchased from Scharlau (Barcelona, Spain).

In the next subsections, methods are briefly described. Further details are provided in the Supporting information file.

2.3. Film preparation

Sixteen grams of zein powder were dissolved in 100 mL of a hydroalcoholic solution (80% *v*/v) at 80 °C. Fifteen grams of plasticizers (glycerol or oleic acid) per 100 g of zein were added to the cool zein solution. Finally, LAE was added to the film forming solution at concentrations of 5 or 10% (*w*/w with respect to zein content). Forty micrometer thick films were obtained by casting using a 250 μm deep thread spreading bar (Lin Lab, Logroño, Spain) and a force air-drying tunnel.

Polypropylene/active zein bags were prepared by spreading the above zein solution onto a corona-treated polypropylene film.

2.4. Film characterization

2.4.1. Mechanical properties

Tensile strength (TS), percentage of elongation at break (EB) and Young's modulus (YM) were measured using a Mecmesin model MultiTest 1-I universal machine (Landes PoliIbérica, S.L., Barcelona, Spain). The results presented are the averages of at least ten measurements.

2.4.2. Water interaction

The water vapour permeability (WVP) of the films at 75% RH was determined gravimetrically following the ASTM standard test method (ASTM, 2010). Water solubility was determined by immersion in a phosphate buffer (pH = 7) for 24 h at room temperature, and calculating the film weight loss. The results are the average of three replicates.

2.4.3. Optical properties

The films' colour was determined with a CR-300 Minolta Chroma (Minolta Camera Co., Ltd., Osaka, Japan) and the results were expressed in accordance with the CIELAB system (L*: lightness, a*: red/green, b*: yellow/blue, ΔE: Colour difference (Higueras et al., 2013a)). Film transparency was determined as the transmittance percentage at 660 nm using a UV–visible spectrophotometer (Agilent, 8453, Barcelona, Spain).

2.4.4. LAE release rate from films into food simulating solvents

Samples of zein films with 10% LAE (3 cm^2) previously measured in weight and thickness (40 ± 5 μm) were immersed in 5 mL of acetic acid (3% *v*/v), ethanol (10% v/v), and MilliQ water, selected as food simulants. Release studies were performed at 4 and 37 °C by double side exposure. Released LAE was determined by using an Agilent 1200 series HPLC system equipped with a C18 reversed-phase column (150 mm × 3.9 mm, 5 μm) and a UV detector (205 nm) (Agilent, Barcelona, Spain). The mobile phase was acetonitrile/water acidified with trifluoroacetic acid (0.1%) (50:50), the flow rate 1 mL/min, and the injection volume 20 μL (Muriel-Galet et al., 2014).

2.4.5. Film morphology

Films exposed to these food simulants were dried in a desiccator for 12 h, and immersed in liquid nitrogen where they were cryofractured. The fracture surface was analysed by field emission scanning electron microscopy on the surface of a cryogenic fracture, following the procedure reported by Cerisuelo et al. (2015). Full description of the method can be found in the supporting information.

2.5. Antibacterial assays

Antimicrobial properties of plasticized zein films with 10% LAE were tested against *E. coli* and *L. monocytogenes*. 0.25 g portion of the film (cut into pieces of 1.5 cm^2) were immersed in 10 mL of Tryptone Soy broth containing 10^5 Colony forming units (CFU)/mL in exponential phase. The tubes were then incubated at 37 °C for 24 h. Zein films without active agent were also used in every experiment as control. Finally, depending on the turbidity of samples, serial dilutions were made with peptone water and plated on petri dishes with agar medium. Colony forming units were counted after incubation at 37 °C for 24 h. The experiments were made in triplicate,

2.6. Evaluation of polypropylene active zein bags in chicken soup

Chicken soup was elaborated with the following ingredients and proportions: chicken breast (250 g), dried parsley (40 g), onion (220 g), carrots (100 g), 1 L tap water, and salt (1 g). All these ingredients were purchased from a local market. Chicken breast, sliced onion and water were placed in a stainless steel container and heated until boiling; then carrot cubes, dried parsley and salt were added and cooked for 1 h. The soup was transferred to a sterile jar in sterile conditions and was stored at 4 °C until use.

The chicken soup was packaged in polypropylene/active zein bags (10% LAE). Each active bag was filled with 10 mL of chicken soup and inoculated with 100 µL of *L. monocytogenes* or *E. coli* in exponential phase (10^5 CFU/mL), heat sealed and stored at 4 ± 1 °C for 10 days. Antimicrobial activity was tested in the 10th day after packaging, by performing serial dilutions with peptone and subsequent plating in Palcam Listeria Selective Agar and Brilliant Green Agar for *L. monocytogenes* and *E. coli*, respectively. Plates were incubated at 37 °C for 48 h. Soup bags without LAE were used as controls to check the effectiveness of the antibacterial films against bacteria. All experiments were carried out in triplicate.

2.7. Statistical analysis

Statistical analysis was performed on a completely randomized design with the analysis of variance (ANOVA) procedure using SAS software (Version 9.1). Least significant difference LSD's multiple range tests were used to compare the difference between mean values of films specimen's properties at the level of $P = 0.05$.

3. Results and discussion

During a visual inspection, all zein films were homogeneous, with smooth surfaces, flexible, transparent, easy to handle, slightly yellowish, and without pores or discontinuities. Both plasticizers made the films flexible and the addition of LAE did not visually affect the macroscopic characteristics of the films.

3.1. Mechanical properties of zein based films

Mechanical properties of materials for packaging applications are important, especially in the case of biopolymers due to their fragility (Ghanbarzadeh and Oromiehi, 2008). The tensile strength (TS), Young's modulus (YM) and elongation at break (EB) values obtained for the developed zein films are shown in Fig. 1. Control films plasticized with oleic acid (ZO) presented the highest TS. YM and EB values, significantly higher than control films plasticized with glycerol. Xu et al. (2012) reported similar results indicating that oleic acid plasticized the hydrophobic domains of the matrix, while glycerol plasticized the polar ones, reducing inter-chain hydrogen bonding and subsequently cohesive energy density. On the contrary, Cao et al. (2009) observed a reduction of EB and TS values in gelatine films plasticized with oleic acid, which was caused by a heterogeneous dispersion of the plasticizer in the gelatine matrix, a more hydrophilic water-soluble polymer. In this sense, since oleic acid is more compatible with zein than with gelatine, it is more uniformly dispersed in the polymer matrix, contributing to improve the mechanical behaviour of films.

The incorporation of LAE into the films plasticized with oleic acid caused an unexpected significant reduction in film TS, YM and EB values ($P < 0.05$), without differences caused by the LAE concentration (Fig. 1). Interactions between LAE molecules and oleic acid reducing the plasticizing effect on the biopolymeric matrix or interactions between LAE and the biopolymer could be causing this effect. On the contrary, the incorporation of LAE in films containing glycerol appears to provide an improved plasticization with significant increases of both TS and EB values. The LAE concentration did not show a significant effect on the measured mechanical properties. Some factors such as composition and structure of biopolymer (Motedayen et al., 2013), capacity of active agent to interact with matrices (Atarés et al., 2011), relative proportion of polymer, type of active agent (Atarés and Chiralt, 2016), and preparation conditions (Motedayen et al., 2013) have been reported to modify the mechanical behaviour of hydrocolloid matrices.

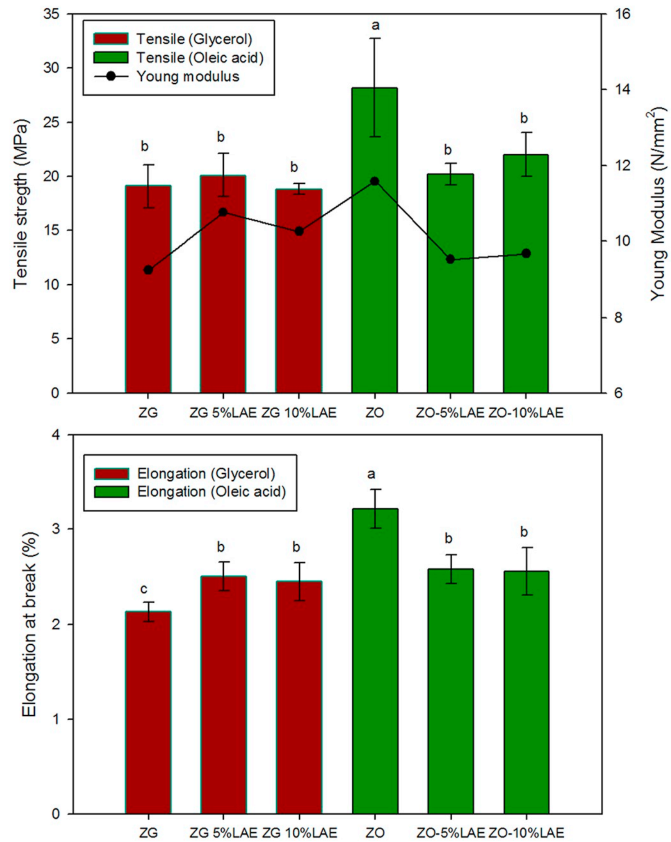

Fig. 1. Effect of plasticizers (glycerol and oleic acid) and LAE concentration on the mechanical properties of zein films: (A), tensile strength (in columns – left y-axis) and Young's modulus (dots – right y-axis); (B) deformation at break. Different letters on top of error bars indicate significantly different values ($P < 0.05$).

3.2. Water-zein film interactions

Most hydrocolloids present high sensitivity to water, sensitivity that affects relevant properties of films with potential application in food packaging, such as partial or full dissolution in water or aqueous products, and high water vapour permeability. Fig. 2 presents the water solubility and water permeability of the developed films. Water solubility (WS) is often used as an important indicator of film resistance to water. As can be seen, water resistance of control oleic acid plasticized films (WS = 1.30 ± 0.08%) was greatly increased with respect to glycerol-plasticized film (12.8 ± 1.4%) in agreement with observations of Ghasemlou et al. (2011b) who found a decrease in the WS of kefiran film by incorporation of OA. This result could be expected from the different water solubility of oleic acid (poor) and glycerol (very high). A comparison between the WS of zein with that of different hydrocolloids such as gelatine (63.81%) (Hosseini et al., 2013), chitosan (31.64%) (Martins et al., 2012) or alginate (99.55%) (Abdollahi et al., 2013) confirm the better potential of zein films as packaging material for aqueous food. This characteristic could be related to zein amino acid composition with more than half of the amino acid residues being nonpolar, including high percentages of leucine (20%), proline (10%), and alanine (10%) (Cabra et al., 2005; Geraghty et al., 1981).

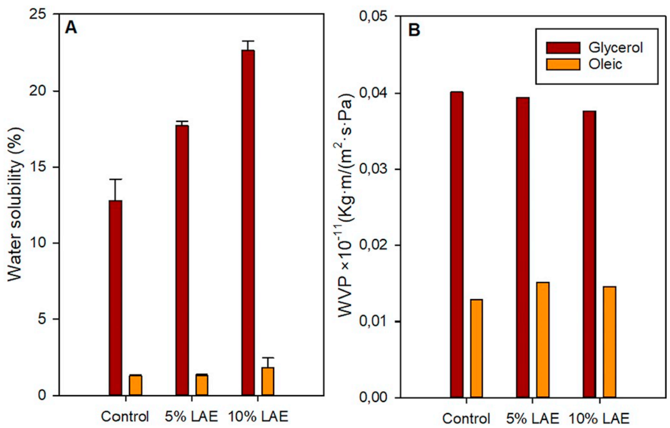

Fig. 2. Interactions between water and the developed zein films. (A), percentage of water solubility; (B), water vapour permeability.

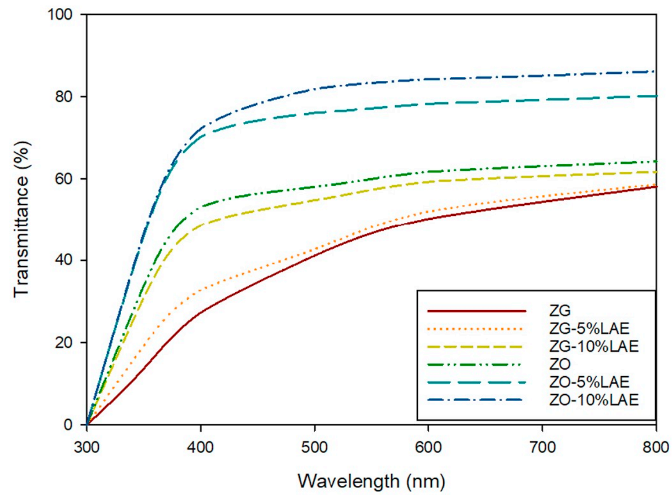

Fig. 3. Transmittance of zein-based films in the UV–visible wavelength range.

When LAE was incorporated in the films, the WS of glycerol-plasticized zein films increased with the concentration of LAE from 12.76 ± 1.76 (0% LAE) to 22.6 ± 0.60% (10% LAE), indicating that the active agent is practically fully released into water. This is in agreement with the large water–oil partition coefficient of LAE (Bakal and Diaz, 2005). A full release of LAE from hydrophilic EVOH to water was also reported elsewhere (Muriel-Galet et al., 2014). This is also in agreement with previous reports on different changes induced in water affinity of bio-films by incorporation of polyols (Ghasemlou et al., 2011a) and LAE (Muriel-Galet et al., 2014; Rubilar et al., 2016). On the contrary, the addition of LAE hardly affected the WS of OA-plasticized zein film (Fig. 2). This could be confirming that some interactions among LAE, oleic acid and protein could be delaying or restricting LAE release from film.

For many food products, a good water vapour barrier provided by the packaging is required to keep quality and safety throughout product shelf life. As can be seen in Fig. 2, the control OA-plasticized films showed the lowest WVP (1.28×10^{-14} kg·m/(m^2·s·Pa)). The presence in the zein matrix of hydrophobic oleic acid molecules reduces the water sorption in the films, and as a consequence the water vapour transmission. Perhaps, the presence of extended hydrophobic domains in the matrix, results in a more tortuous path for water molecules diffusion, also driving to a decrease in WVP. No significant effect of LAE addition was observed in water barrier. Similar WVP reductions through biopolymeric films by incorporation of lipids have been reported (Fabra et al., 2009; Kamper and Fennema, 1984; Taqi et al., 2013; Vargas et al., 2009). On the contrary, the highest WVP was obtained in control glycerol-plasticized films (4.01×10^{-14} kg·m/(m^2·s·Pa). In both cases (oleic acid or glycerol containing films), the active agent concentration hardly affected the WVP of films, although a slight reduction of WVP (about 6%) was observed with 10% LAE in glycerol-plasticized film (Fig. 2). A comparison between the WVP values of zein films with those of commodity synthetic films in food packaging such as polyethylene (Wihodo and Moraru, 2013) revealed that the barrier properties of zein films is much weaker. Nevertheless, zein WVP values are in the range of EVOH ($5.7 \times \cdot 10^{-15}$ kg·m/(m^2·s·Pa)), cellophane (6.8×10^{-14} kg·m/(m^2·s·Pa)) or polylactic acid PLA (5.2×10–$^{-13}$ kg·m/(m^2·s·Pa)) (Duan et al., 2013; Massey, 2003).

3.3. Optical properties of zein based films

Colour properties of film packages can directly affect food appearance and consumer acceptance. The apparent colour properties of the zein films were determined using the CIELAB colour system and the results are shown in Table 1. The high values of luminosity (L* > 88) are indicative of good lightness of all developed zein films. Luminosity (L*) of the films was increased with the incorporation of LAE. On the other hand, slightly negative values of a* and positive values of b* are indicative of a yellowish coloration, despite the zein utilized in this study is a decolorized and deodorized product. The total colour of active plasticized films showed significant differences ($P < 0.05$) compared to neat plasticized films.

Transparency is another relevant property of films for food packaging applications. As shown in Fig. 3, all the neat plasticized zein films were highly transparent when exposed to wavelengths between 400 and 700 nm. Nevertheless, these films are also partially transparent to UV irradiation, which may affect foodstuff quality due to lipid oxidation, vitamin loss, discoloration, and off-flavours (Martins et al., 2012). The light transmittance at 280 nm of neat OA-plasticized and glycerol-plasticized films were 0.93 and 0.16%, respectively, revealing a great UV-light blocking properties of zein. With the addition of LAE, the transparency decreases at all wavelengths of the UV–visible spectrum. The highest light-blocking effect was observed when active agent content was 10% in OA-plasticized zein film.

3.4. Antimicrobial agent release of zein based films

The kinetics of LAE release from plasticized-zein films were

Table 1
Colour (in CIELAB coordinates, L*, a*, b*, C*, H and ΔE) and transparency (%) of the developed zein films as affected by plasticizers and LAE concentration (% *w*/w).

Film	L*	a*	b*	C*	H	ΔE	Transp (%)
ZG	88.3 ± 0.1^b	−1.87 ± 0.02^a	6.7 ± 0.1	6.9 ± 0.1	105.7 ± 0.1	–	62.9 ± 0.8^a
ZG 5%LAE	90.1 ± 0.1^a	−1.74 ± 0.07^b	6.3 ± 0.2	6.6 ± 0.2	105.4 ± 0.2	8.5 ± 1.0	59.0 ± 0.1^b
ZG 10%LAE	90.19 ± 0.4^a	−1.81 ± 0.07^{ab}	6.5 ± 0.2	6.8 ± 0.1	105.6 ± 0.1	8.7 ± 1.1	58.9 ± 0.6^b
ZO	88.0 ± 0.5^b	−1.84 ± 0.08^{ab}	7.0 ± 0.7	7.2 ± 0.7	105.8 ± 0.9	–	64.2 ± 0.2^a
ZO-5%LAE	90.4 ± 0.3^a	−1.75 ± 0.06^b	6.3 ± 0.2	6.6 ± 0.2	105.4 ± 0.4	8.2 ± 0.4	56.4 ± 1.1^b
ZO-10%LAE	90.4 ± 0.5^a	−1.74 ± .02^b	6.5 ± 0.2	6.7 ± 0.2	105.1 ± 0.3	8.3 ± 0.4	56.7 ± 0.7^b

Different letters in column indicate significantly different values ($P < 0.05$).

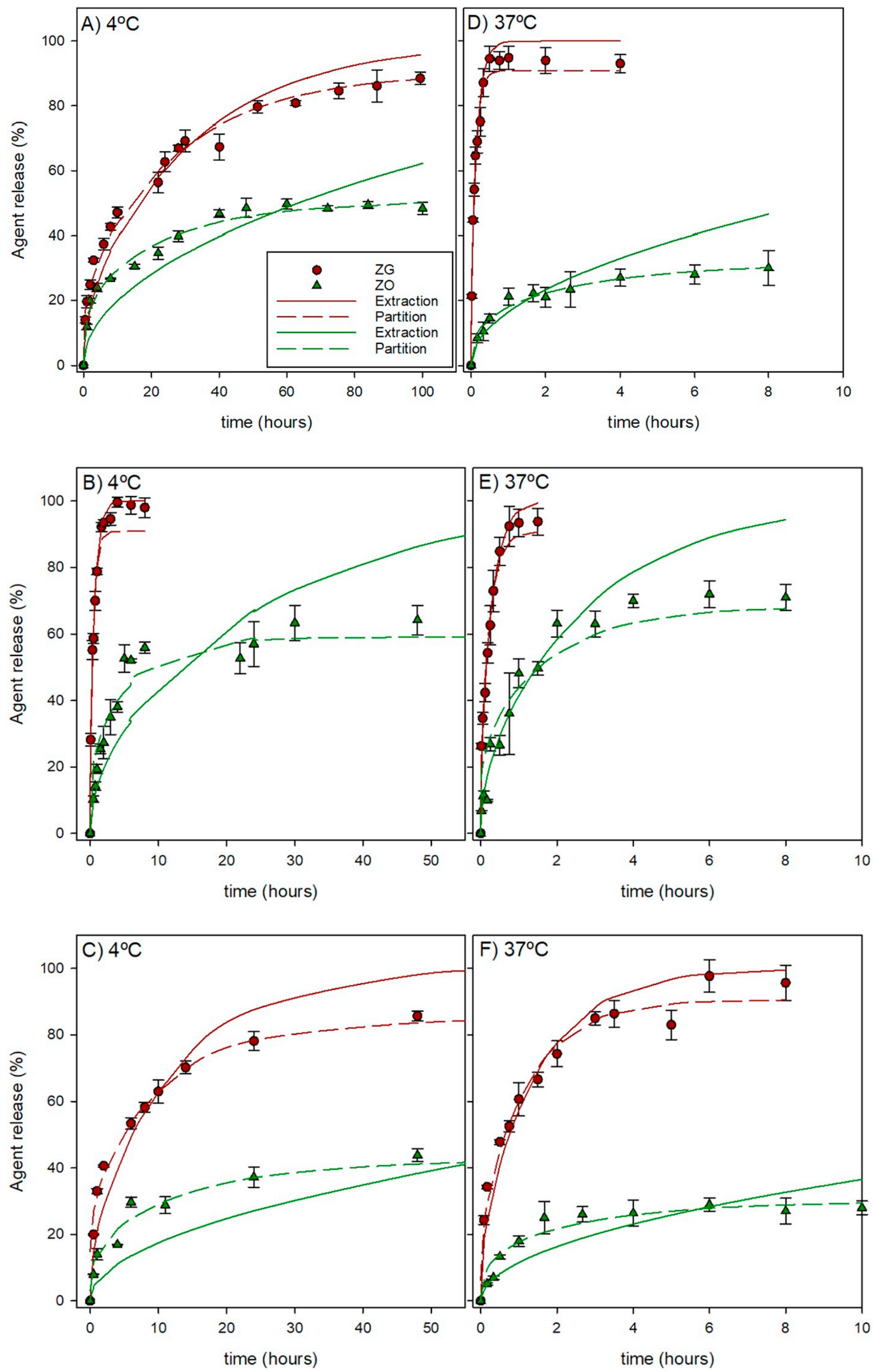

Fig. 4. Kinetics of LAE release from plasticized-zein films at 4 and 37 °C by immersion in 10% ethanol (A and D), 3% acetic acid (B and E), and deionized water (C and F). Symbols correspond to experimental data, lines are theoretical curves obtained by curve fitting: dashed lines, fitting to Eq. (1) and full lines, fitting to Eq. (2).

determined at 4 and 37 °C by immersion in ethanol (10% *v*/v), acetic acid (3% v/v), and deionized water. The obtained data are presented in Fig. 4. As can be seen in all tests, films plasticized with glycerol showed a faster and more extended release than films plasticized with oleic acid.

At 4 °C, the obtained results show great differences in the release of the active compound, being affected by the polymer-retention capacity and the polymer-liquid medium interactions. The more extended release was observed for glycerol plasticized films in acetic acid (ca. 100%), followed by the release in ethanol 10% (ca. 90%) and finally, in water (81%) (Fig. 4A–C). Kinetically, the faster process was observed in acetic acid as well, reaching 50% release in 20 min of exposure, much faster than in water and ethanol (5 and 15 h). The morphology of the films after the exposure to the different simulants was analysed by SEM. Fig. 5 shows the surface morphology of glycerol plasticized films after immersion in the diverse simulants at 4 °C. As can be seen, the surface is coarse in all cases probably due to the swelling effect of the water absorbed in the film and the release of LAE and glycerol. Nevertheless, a rougher surface was observed for the film in contact with 3% acid acetic, indicating a greater interaction and swelling of the biopolymeric

Fig. 5. SEM images obtained from the cryo-fracture surface of the glycerol plasticized active zein film, after exposure to various food simulants (Ethanol (10%), Acid acetic (3%), and Deionized water) at 4 °C.

Table 2
Values of the diffusion coefficient and the partition coefficient obtained by fitting of the experimental values to Eqs. (1) and (2).

Sample	Extraction process, Eq. (2)		Partition process, Eq. (1)			
	D (m^2/s)	r^2	K	% Eq.	D (m^2/s)	r^2
10% ethanol						
ZG 4 °C	1.34×10^{-15}	0.98	45	90.3	1.40×10^{-15}	0.99
ZO 4 °C	3.45×10^{-16}	0.68	413	50.2	9.80×10^{-16}	0.98
ZG 37 °C	2.71×10^{-13}	0.98	42	90.9	3.53×10^{-13}	0.99
ZO 37 °C	2.37×10^{-15}	0.36	906	31.5	6.72×10^{-15}	0.88
3% acetic						
ZG 4 °C	6.38×10^{-14}	0.99	21	95.1	8.32×10^{-14}	0.99
ZO 4 °C	1.67×10^{-15}	0.78	288	59.1	4.01×10^{-15}	0.94
ZG 37 °C	1.48×10^{-13}	0,99	42	90.9	1.75×10^{-13}	0.98
ZO 37 °C	1.50×10^{-14}	0,96	292	58.8	2.02×10^{-14}	0.96
Water						
ZG 4 °C	3.52×10^{-15}	0,95	78	84.3	3.04×10^{-15}	0.97
ZO 4 °C	2.67×10^{-16}	0,80	568	42.3	1.49×10^{-15}	0.97
ZG 37 °C	2.89×10^{-14}	0,98	44	90.5	3.27×10^{-14}	0.98
ZO 37 °C	1.76×10^{-15}	0,89	791	34.5	5.12×10^{-15}	0.95

Table 3
Antimicrobial effectiveness of zein based films with 10% LAE against *L. monocytogenes* and *E. coli*, expressed as logarithm of colony forming units per milliliter (Log (CFU/mL)) and log reduction value (LRV)*.

	L. monocytogenes		*E. coli*	
	Log (CFU/ml)	LRV	Log (CFU/ml)	LRV
Zein-gly-control (A)	8.46 ± 0.05		9.19 ± 0.05	
Zein-gly-10%LAE	3.47 ± 0.37	4.99[a]	5.02 ± 0.19	4.16[a]
Zein-OA-control (B)	8.89 ± 0.02		8.98 ± 0.13	
Zein-OA10%LAE	8.68 ± 0.07	0.21[b]	8.87 ± 0.16	0.11[b]

Different letters in column indicate significantly different values ($P < 0.05$).

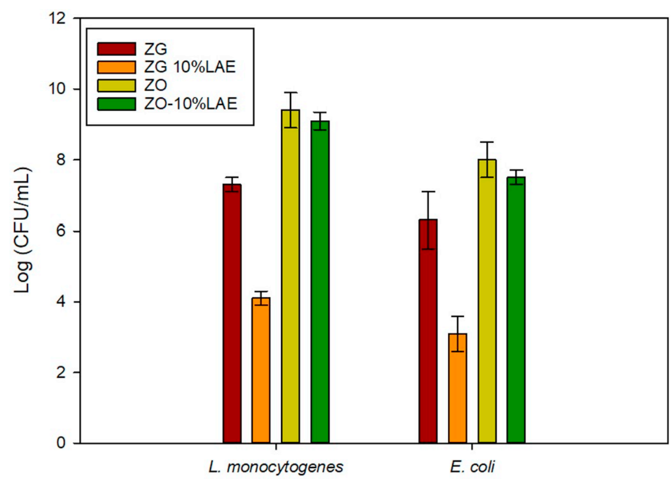

Fig. 6. Antimicrobial activity of PP/10% LAE-zein bags against *L. monocytogenes* or *E. coli* inoculated in chicken soup after 10 days of storage.

matrix. This swelling increases free volume and eases molecular diffusion, which could be related to the much faster release observed.

Similar behaviour was observed at 37 °C although in all cases the kinetics are faster as could be expected since temperature accelerates diffusional processes. For instance, the releases in ethanol and acetic acid reached equilibrium in 2 h. Such a fast release might be interesting to reach rapidly a high antimicrobial concentration when the product is exposed to high temperatures, but also, such rapid release may reduce long-term food protection in the surface of the foodstuff (Appendini and Hotchkiss, 2002). Since, active antimicrobial packaging of not sterilized products is generally combined with refrigeration (Labuza and Breene, 1989) the release rate of active agent at 4 °C is time-extended, assuring safety and quality levels during storage (Franssen et al., 2004).

As a first approximation, Fick's laws can be used to analyse kinetics of the releasing process. For reasons of simplicity, several assumptions were considered: the active agent is homogenously distributed in the matrix at $t = 0$; the polymer membrane had a constant thickness L; and the agent released was instantaneously distributed into a limited volume of simulant. With these boundary conditions, the process can be described by:

$$\frac{m(t)}{m_0} = \frac{\alpha}{1+\alpha}\left\{1 - \sum_{n=1}^{\infty}\left[\frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} exp\left(-\frac{4Dq_n^2 t}{L^2}\right)\right]\right\}$$
$$\alpha = \frac{V_S}{V_P K};\ \tan(q_n) = -\alpha q_n \qquad (1)$$

where m(t) is the mass of agent released at time t, m_o is the total amount of agent initially in the sample, L is the thickness, V_P and V_S are the volume of polymer and simulant, respectively, K is the partition coefficient defined as the ratio between the agent concentration in the film and in the simulant at equilibrium, and D is the diffusion coefficient of the agent in the polymer matrix.

Considering that the agent is fully released into the simulant, then the conditions of transport can be simplified, assuming that the simulant is infinite or that K = 0. Under these conditions, Fick's law solution can be described by:

$$\frac{m(t)}{m_0} = 1 - \sum_{n=0}^{\infty}\left(\frac{8}{(2n+1)^2\pi^2} exp\left[\frac{(2n+1)^2\pi^2 Dt}{L^2}\right]\right) \qquad (2)$$

Table 2 collects the transport parameters obtained by curve fitting using the above equations and the Solver function of Excel from

Microsoft. Also, the theoretical curves are included in Fig. 4. As can be seen, the experimental data were well described by the extraction process (Eq. (2)) for the samples plasticized with glycerol but failed to describe the results observed for oleic acid samples. Eq. (1) that describes the partition process matches very well the experimental data for all samples. For the glycerol samples, the partition coefficient values were low, below 100 and the release at equilibrium reached in all cases percentages ca. 90%. The diffusion coefficient values were similar to those obtained with Eq. (2). On the contrary, for the samples plasticized with oleic acid, the percentage of released agent was significantly lower, between 30 and 60%. The D values obtained with Eq. (1) were in general much higher than those obtained with Eq. (2), the greater the K value, the larger the difference, as Eq. (2) fails to describe the process (see Fig. 4). Considering only the D values obtained for the partition process, the D value is always higher for glycerol than for oleic acid samples under the same temperature/simulant condition. The hydrophilic nature of glycerol may increase and accelerate the swelling of the zein matrix exposed to the three aqueous liquids. On the contrary, the incorporation of oleic acid decreases these processes as already commented in previous sections. Comparing the effect of simulants, the exposition to ethanol resulted in the slower release, followed by exposition to water and acetic acid. This latter simulant causes the greater swelling in the matrix and correspondingly, the faster diffusion of LAE in the matrix.

Previous studies showed very fast release rate of LAE from chitosan (Higueras et al., 2013b) and EVOH (Muriel-Galet et al., 2014). However, the controlled release of LAE from zein film, especially when plasticized with oleic acid, might provide a better protection of food products over time.

3.5. Antimicrobial properties of active zein based films

The antimicrobial properties of plasticized-zein films incorporated with 10% LAE were analysed in vitro and the results are presented in Table 3. Plasticized zein films without LAE were used as control (a previous analysis proved that unplasticized zein films had no bactericidal or bacteriostatic effect). As shown in Table 3, the log reduction of active glycerol-plasticized film against *L. monocytogenes* and *E. coli* was 4.99 and 4.16, respectively. However, active OA-plasticized films showed 0.21 and 0.11 log reduction against *L. monocytogenes and E. coli*, respectively. A less effective material could be expected from the lower release values measured in the previous section. However, the LAE release reached in water at 37 °C ca. 80% of the total content and some growth inhibition could be foreseen. This reduction of effectiveness could be attributed to electrostatic interaction of oleic acid (negative charge) with LAE (cationic charge) that tightly entrapped in zein matrix, according to other authors' reports (Asker et al., 2011; Bonnaud et al., 2010; Dai et al., 2016; Loeffler et al., 2014). However, the inactivity of the released LAE molecules could be attributed to the surfactant characteristics of LAE that might suggest that these molecules could be involved in interactions with oleic acid in the liquid media, and not free to interact with the microorganisms, limiting their action and their efficiency.

3.6. Antimicrobial activity of bilayer films in a real food packaging system

Fig. 6 shows the antimicrobial properties of different polypropylene PP/ LAE zein bags filled containing chicken soup inoculated with *L. monocytogenes* and *E. coli* at 4 C° on 10th days of storage. As can be seen, the PP/LAE glycerol-plasticized zein bags produced a reduction of 3.21, and 3.07 log against *L. monocytogenes* and *E. coli*, respectively. On the contrary, the results for the PP/LAE OA-plasticized zein film shown no antimicrobial effect, clearly confirming the insufficient antimicrobial activity to inhibit bacteria growth revealed in the in vitro test (see Table 2). Comparing the obtained log reduction values (LRV) with recent works with zein films containing *Zatariamultiflora Boiss.* essential oil (ZEO) as active agent (Kashiri et al., 2017), the incorporation of LAE in the glycerol-plasticized zein films is more effective than ZEO to control growth of both pathogenic bacteria. Finally, although the plasticization of zein with oleic acid provided very relevant improvement of optical, barrier and mechanical properties, the interaction with LAE results in an inefficient active packaging material, with lower levels of agent release, and with lower activity of the released agent.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108272.

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