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Incorporation of organic acids turns classically brittle zein films into flexible antimicrobial packaging materials

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Abstract

This study aimed to turn classically brittle zein films into flexible antimicrobial ones by the use of lactic (LA), malic (MA) and tartaric acids (TA). The most effective plasticizer was LA (400% elongation at break [EB] at 4%), while MA (189% EB at 4.5%) and TA (68% EB at 5%) showed moderate and limited plasticizing effects, respectively. The LA- and MA-loaded films maintained their flexibility during 30-day storage at 4°C or 25°C. Fourier transform infrared (FTIR) analysis suggested that the plasticization of LA and MA could be related to secondary structural changes in zein such as increased α-helix and random coils (mainly by MA) and spaced/modified intermolecular (only by LA) and intramolecular (mainly by MA) β-sheets. Atomic force and scanning electron microscopy showed that LA and MA gave more homogenous and smoother films than TA. Films with LA showed the highest water vapour permeability followed by those of control, MA- and TA-loaded films. Films with 3%–4% LA or MA formed clear zones on *Listeria innocua* and *Klebsiella pneumonia*, but only films with LA formed clear zones on *Escherichia coli*. All OA-loaded films gave unclear zones on *Staphylococcus aureus* in disc-diffusion tests, but this bacterium was inactivated rapidly in antimicrobial tests based on surface inoculation tests. LA is the best OA to develop flexible antimicrobial films from zein, an industrial by-product that films could not have been utilized as a widespread packaging material due to their brittleness.

KEYWORDS
antimicrobial, edible film, flexible, organic acid, zein

1 | INTRODUCTION

Zein, a hydrophobic prolamin group storage protein that forms 23%–50% of corn proteins,[1–3] attracts particular industrial interest since it is the major co-product of the rapidly growing oil and bioethanol industries.[4,5] There are four different molecular fractions of zein (α, β, γ and δ-zein), but α-zein is the most abundant one that forms almost 80% of total zein fractions.[6,7] Zein is already used as a coating material for candies, fresh and dried fruits, and nuts.[1,8,9] However, continuous efforts have been spent to improve zein extraction processes and film properties and to use this protein as a more widespread film-forming biopolymer.[10–12]

The most promising properties of zein as a biopolymer are as follows[1]: outstanding ability to form packaging (films and coatings) and encapsulating (nanofibers and nanoparticles) materials,[2] solubility in organic solvents like ethanol,[3] compatibility and sustained release properties of its materials with natural antimicrobials and antioxidants, and[4] suitable gas permeability characteristics of its coatings applied to fresh fruits and vegetables.[10,13,14] In contrast, the highly brittle nature of zein films upon drying by storage is the major disadvantage that interferes with the use of this biopolymer as a universal packaging material.[15] Therefore, plasticization studies have become one of the challenging topics in the field of zein research.[16] The characteristic film structure of zein consists of a meshwork composed of doughnut

structures formed by asymmetric rods joined to each other.[17] The zein film integrity is maintained mainly by hydrophobic interactions together with strong intermolecular disulphide bonds that keep the zein rods together.[17,18] Thus, the brittle nature of zein films is attributed to their extreme hydrophobicity and rigid structure that prevents their sufficient and proper interactions with plasticizers.[4] Different researchers have developed alternative methods and formulations to overcome the brittleness problem of zein films. For example, Ghanbarzadeh et al.[19] employed different sugars such as glucose, galactose and fructose for the plasticization of zein. Sessa et al.[20] modified zein film networking by crosslinking with glutaraldehyde and glacial acetic acid. Lawton[21] tested dibutyl tartrate, triethylene glycol, polyethylene glycol (300), levulinic acid, glycerol and oleic acid as zein plasticizers. Xu et al.[22] showed the synergistic plasticizing effect of oleic acid and glycerol on zein film while Huo et l.[23] successfully tested combinations of glycerol and polyethers to increase the flexibility of zein films. Arcan and Yemenicioğlu[24] followed a different principle in zein film plasticization by applying bioactive polyphenols such as gallic acid, p-hydroxybenzoic acid, ferulic acid, catechin, flavone and quercetin as plasticizers. The strategy of employing bioactive compounds for zein plasticization is highly advantageous since polyphenols provide not only the desired flexibility but also antimicrobial and antioxidant activities.[25] Boyacı et al.[26] used the same strategy for essential oils such as eugenol, carvacrol and thymol to plasticize zein films and obtain antimicrobial coating materials against plant pathogens. However, the applicability of polyphenols has some limitations originating from their strong flavour and aroma characteristics. The use of organic acids (OAs) in the plasticization of edible films may also provide additional benefits such as antimicrobial and antioxidant activities. In the literature, different OAs are used for the plasticization of edible or biodegradable films. For example, it was reported that the ascorbic acid is an effective plasticizer for starch–polyvinyl alcohol (PVA)[27] and chitosan films[28] while malic acid (MA) was reported to act as a plasticizer for gelatin films[29] and sorbic acid for whey protein films.[30] The use of OAs like lactic acid alone[31] or in combination with triethylene glycol–sorbitol[32] has also been tested for plasticization of zein films. Lactic and acetic acids were also employed to increase the extensibility of zein doughs.[33] However, studies related to the characterization of the plasticization mechanism of zein films with different OAs, and their effects on zein film properties and antimicrobial activity are scarce.

The aim of the current work was to conduct a detailed characterization study to understand the effects of incorporating OAs such as lactic (LA), MA and tartaric acids (TA) in major properties (e.g., mechanical, morphological and water vapour barrier properties) and antimicrobial activity of zein films. The study targeted to obtain not only zein films highly flexible at different storage conditions but also films having antimicrobial effects on selected major pathogenic Gram-positive (*Listeria innocua* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). This work is important since it provides an alternative approach to evaluate zein not only as a flexible but also as an antimicrobial packaging material. The developed films could find different food applications since

selected OAs are generally recognized as safe (GRAS) compounds that are already employed extensively as an acidulant and antimicrobial agent in different food products.

2 | MATERIALS AND METHODS

2.1 | Materials

Zein was obtained from Sigma-Aldrich (St. Louis, MO, USA). TA and glycerol were obtained from Merck (Darmstadt, Germany). L-lactic acid 85% (LA) and MA were obtained from SAFC (Germany) and Sigma-Aldrich (CHEMIE GmbH, Germany), respectively. The test bacteria, *L. innocua* (NRRL-B 33314), *S. aureus* (ATCC 29213), *E. coli* (NRRL B-3008) and *K. pneumoniae* (ATCC 10031), were kindly provided by the culture collection of the microbiology laboratory in the Department of Food Engineering at Izmir Institute of Technology.

2.2 | Methods

2.2.1 | Film making

The zein film was produced as described in Padgett et al.[34] Initially, zein (1.4 g) was dissolved in 8.2 ml of ethanol (96%) by mixing for 25 min at 200 rpm. The beaker was covered with a Parafilm® M (Bemis NA, Neenah, WI) to avoid the evaporation of ethanol. Glycerol (0.4 ml) was then added dropwise to the zein solution and mixed for 5 min. The use of glycerol is essential to obtain self-standing control films that could be peeled off from casting templates. Thus, the same amount of glycerol was added to all film formulations. After the addition of glycerol, the film solution was heated on a hotplate under continuous stirring until boiling. At this point, mixing was ceased, and the film solution was cooled to room temperature. Different OAs (LA, MA and TA) were then added to zein-glycerol solution at different concentrations, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% or 5% (w/w of film forming solution). The mixtures were then homogenized (Heidolph®, Silent Crusher M, Germany) at 10,000 rpm for 4 min, and 4.3 g of the homogenate from each formulation was cast into glass templates (W × L × H: $8.5 \times 8.5 \times 0.4$ cm^3). Finally, films were dried at 25°C for 24 h using a standard incubator. Films were stored for 30 days under room temperature (25°C) or under refrigeration (4°C) in order to monitor changes in their mechanical properties during storage. Samples were analysed at 0, 10, 20 and 30 days.

2.2.2 | Mechanical properties of films

Tensile strength (TS), elongation at break (EB) and Young's modulus (YM) of films were determined using a Texture Analyzer (TA-TX2, Stable Microsystems, Godalming, UK) according to ASTM Standard Method D 882-02.[35] The films were cut into strips (12 mm in width and 80 mm in length) before tests. The initial grip distance was

30 mm, and the crosshead speed was 50 mm/min. At least five measurements were conducted for each film. Film thickness was measured with a micrometre (Chronos®, UK) by conducting three measurements for each film.

2.2.3 | FTIR analyses of films

For Fourier transform infrared (FTIR) analysis, the zein films were placed on the horizontal attenuated total reflectance sampling accessory (ZnSe crystal plate) of a FTIR (Spectrum 100 Instrument, Perkin–Elmer Inc., Wellesley, MA). The FTIR spectra were recorded with 32 scans at 4-cm^{-1} resolution from 4,000- to 650-cm^{-1} wavenumbers. The background spectrum was automatically subtracted from the spectra of the samples. Individual components within the spectra were estimated by second derivative and Lorentz curve fitting in the regions of 1,700–1,600 cm^{-1} using OriginPro 9.0 software (OriginLab, USA).[36,37] The area under the curve of each deconvoluted band was calculated. All the deconvolutions were done with an R^2 of 0.99. The wavenumber ranges of secondary structures were described as follows; intermolecular β-sheets at 1,610–1,625 and 1,685–1,695 cm^{-1}, intramolecular β-sheets at 1,630–1,640 and 1,670–1,684 cm^{-1}, random coil at 1,640–1,648 cm^{-1}, α-helices at 1,648–1,658 cm^{-1} and β-turns at 1,660–1,668 cm^{-1}.[38-40]

2.2.4 | AFM of films

The surface images of control films and films with 3.5% LA, MA or TA were carried out by an atomic force microscop (AFM) (MMSPM Nanoscope 8 from Bruker, USA) in an intermittent-contact mode in the air with silicon tips (resonance frequency ≈340 kHz, spring constant ≈40 N m^{-1}, tip radius 8 nm). The captured images (min 3 for each sample) were analysed by Nanoscope Analysis software v.1.5 (Bruker, USA). The surface roughness R_{rms} was calculated as the root mean square average of height deviations (Z_i) taken from a mean data plane ($\underline{Z}$).

$$R_{rms} = \sqrt{\frac{\sum_{i=1}^{N}(Z_i - \underline{Z})^2}{N-1}}.$$

The R_{max} parameter indicates the maximum vertical distance between the highest and the lowest points in the image.

2.2.5 | SEM of films

The cross-sectional morphologies of control films and films with 3.5% LA, MA or TA were determined by using scanning electron microscop (SEM) (Philips XL 30S FEG, FEI Company, Netherlands) under high vacuum mode at an operating voltage varying between 2 and 3 kV. The films were placed into liquid nitrogen for fast freezing and crashed for the SEM examination. After that, the samples were gold coated with a sputter coater (Emitech K550X, Quorum Technologies Inc., UK) under 15 mA for 1 min. The thickness of the films was measured from SEM cross-sectional views of films from 500× magnified images.

2.2.6 | WVP of films

The water vapour permeabilit (WVP) of control films and films with 3.5% LA, MA or TA were measured using Payne permeability cups (Elcometer 5100, England) according to the ASTM Standard Method E96.[41] Each cup was filled with 3 g of dried silica beads. The thicknesses of the samples were measured. Each film sample with a diameter of about 6 cm was cut and placed on top of the cups and sealed with three tight clamps after putting the O-ring. The cups were weighed, and they were placed in a controlled test cabinet (TK 120, Nüve, Turkey) at 25°C and 50% RH. The cups were weighted periodically for 72 h. The weight increase of the cups was plotted against time and the linear portion of the curve with at least five data points with $R^2 \geq 0.99$ was taken for calculation of WVP according to the following equation:

$$WVP = \frac{G L}{A t S (R_1 - R_2)},$$

where G is the weight change from the straight line (g), L is the thickness of the film (mm), t is the time (day), A is the test area (m^2), S is the saturation vapour pressure at test temperature (3.169 kPa at 25°C), R_1 the relative humidity of the test chamber (50%) and R_2 the relative humidity in the dish (0%). Four independent tests per film were performed.

2.2.7 | Antimicrobial activity of films based on the disc-diffusion method

The antimicrobial activity of LA- and MA-containing films was tested at aseptic conditions on agar surface using classical disc-diffusion method against *L. innocua*, *E. coli*, *S. aureus* and *K. pneumoniae*. Nine discs (1.3 cm in diameter) were obtained from each film using a cork borer under aseptic conditions. During tests, discs were placed carefully into Petri dishes containing nutrient agar previously inoculated by spreading the inoculum (0.1 ml). The inoculums were prepared in nutrient broth using an overnight culture of bacteria in aerobic conditions at 37°C. The cultures were then set to 0.5 McFarland with 0.1% peptone water before being used for the antimicrobial tests. The inoculated Petri dishes containing the film discs were incubated for 24 h at 37°C. The diameters of fully formed clear zones that showed strong inhibition were measured by a digital calliper (TorQ, 150 mm, PRC), and average results were expressed in mm^2. The unclear zones that extensive regrowth observed within formed clear zones were not measured, but the presence of microbial growth below discs was checked and noted as a sign of limited antimicrobial activity (LA). No-

zone formation (NZ) around discs was also reported to show the lack of antimicrobial activity.

2.2.8 | Antimicrobial activity of films based on surface inoculation method

The antimicrobial activities of LA- and MA-containing films were performed against *E. coli* and *S. aureus* as described by Boyacı et al.[26] by modifying the isolation medium. The current work was performed using 0.1-M K-phosphate buffer at pH 7.2 instead of peptone water used in the original method to prevent sharp drops in pH caused by OAs in an isolation medium. The microbial load of the inoculated OA-loaded zein films was determined for freshly prepared samples (0 day), and samples kept at room temperature for 1 and 7 days by the spread plate method in triplicate onto nutrient agar. The plates were incubated at 37°C for 24 h, and the colonies were counted in triplicate plates. Microbiological counts were expressed as colony-forming unit per gram (CFU g^{-1}) of each film. The film without OAs was considered the control film.

2.2.9 | Statistical analysis

All the experiments were done in duplicates except for WVP. Results were analysed for significance by using variance analysis (one-way ANOVA) and Fisher post-test using Minitab (ver.16.2.0.0, Minitab Inc., UK). The differences were considered significant if $p \leq 0.05$.

3 | RESULTS AND DISCUSSIONS

3.1 | Effect of different OAs on mechanical properties of films

The effects of LA, MA and TA on the mechanical properties of zein films were displayed in Table 1. It is clear that the addition of all OAs caused a concentration-dependent increase in the EB of films. However, films with 2 and 2.5% (w/w) LA showed 8.3 and 7.7, and 12.7- and 21.8-fold higher EBs than films with MA and TA at the same concentrations, respectively. The highest EBs for each OAs were 400% for films with LA at 4% (w/w), followed by 189% for films with MA at 4.5% and 68% for films with TA at 5%. Apparently, LA caused more increase in film flexibility than other OAs at lower concentrations. Thus, the most effective plasticizer was found as LA, while MA showed moderate, and TA showed the most limited plasticizing effects. In contrast, the increase of film flexibility by OAs caused a concentration-dependent reduction in TS and YM of films. For films loaded with OAs, the highest TS and YM values were obtained for TA-containing films followed by films with MA and LA. However, all OA-loaded films showed significantly lower YM than control zein films ($p \leq 0.05$). Besides, films with 2% and 2.5% TA showed significantly

TABLE 1 Mechanical properties of zein films plasticized with different organic acids

Organic acid	Conc. (%) w/w	Elongation at break (%)[a]	Young's modulus (MPa)	Tensile strength (MPa)	Film thickness (μm)
Control	—	1.83 ± 0.27[I]	3.81 ± 0.18[A]	7.93 ± 0.83[CD]	130 ± 5[E]
LA	2.00	40 ± 15[H]	1.24 ± 0.07[E]	3.73 ± 0.51[FGH]	139 ± 9[CDE]
	2.50	73 ± 21[F]	0.67 ± 0.11[F]	2.60 ± 0.27[I]	143 ± 5[BCD]
	3.00	94 ± 14[E]	0.45 ± 0.07[GH]	1.66 ± 0.06[J]	146 ± 2[BC]
	3.50	249 ± 19[B]	0.13 ± 0.03[IJ]	1.12 ± 0.06[JKL]	146 ± 2[BC]
	4.00	400 ± 43[A]	0.02 ± 0.01[J]	0.49 ± 0.05[M]	147 ± 3[BC]
MA	2.00	4.88 ± 0.66[I]	2.26 ± 0.07[CD]	7.34 ± 0.42[E]	158 ± 9[A]
	2.50	9.43 ± 0.97[I]	2.10 ± 0.08[D]	7.53 ± 0.11[DE]	146 ± 7[BC]
	3.00	33 ± 6[H]	1.18 ± 0.10[E]	3.19 ± 0.30[H]	142 ± 4[BCD]
	3.50	85 ± 12[EF]	0.58 ± 0.07[FG]	2.44 ± 0.17[I]	139 ± 5[CDE]
	4.00	126 ± 15[D]	0.27 ± 0.04[HI]	1.56 ± 0.08[JK]	140 ± 4[CDE]
	4.50	189 ± 30[C]	0.14 ± 0.05[IJ]	1.03 ± 0.09[KLM]	146 ± 8[BC]
	5.00	174 ± 29[C]	0.11 ± 0.02[IJ]	0.75 ± 0.08[LM]	147 ± 12[BC]
TA	2.00	3.17 ± 0.46[I]	3.27 ± 0.25[B]	9.08 ± 0.83[B]	134 ± 3[CDE]
	2.50	3.34 ± 0.35[I]	3.29 ± 0.32[B]	9.70 ± 0.84[A]	119 ± 8[F]
	3.00	5.66 ± 1.35[I]	2.23 ± 0.17[CD]	7.43 ± 0.69[DE]	144 ± 3[CD]
	3.50	6.63 ± 0.87[I]	2.34 ± 0.13[C]	8.22 ± 0.49[C]	137 ± 5[CDE]
	4.00	51 ± 10[GH]	1.25 ± 0.04[E]	3.95 ± 0.23[F]	147 ± 9[BC]
	4.50	52 ± 8[GH]	1.24 ± 0.13[E]	3.88 ± 0.19[FG]	140 ± 2[CDE]
	5.00	67 ± 17[FG]	1.13 ± 0.16[E]	3.33 ± 0.30[GH]	152 ± 9[AB]

[a]Values at each column indicated by different letters are significantly different ($p \leq 0.05$).

higher TS than control zein films ($p \leq 0.05$) while films with 2.5% MA, and films with 3% and 3.5% TA had similar TSs with control zein films ($p > 0.05$). These results suggested that TA acted as a weak plasticizer since it failed increasing spacing and/or it increased networking among joint asymmetric zein rods forming the main film matrix.[17] The capacity of plasticizers to fulfil their function is related to their ability to diffuse within the film matrix and bind onto the biopolymer's surface mainly via H-bonds to increase the free volume of the film matrix.[16,42] The main H-bonding groups of protein include peptide carbonyl, amine, and side-chain hydroxyl, carboxyl (ionized or unionized), and amide groups,[43] while OAs contain carboxyl and hydroxyl groups (Figure 1). The dramatic reductions in TS of films by the addition of LA that contains one —COOH and one —OH group indicate that this OA cannot create extensive intermolecular H-bonds among zein rods that form the film matrix. Instead, it appears that the LA binds mainly intramolecular locations in zein rods and increases intermolecular spacing among them. The MA that has two —COOH and one —OH (MW: 134 Dalton) and TA that has two —COOH and two —OH should also have formed H-bonding with zein biopolymer. However, significant differences between EB and TS values of TA and MA suggested that the additional —OH group of TA caused the formation of extensive intermolecular crosslinking among zein rods and the formation of more rigid films than those of MA. Moreover, it is also important to note that the differences in MWs of OAs could also be a factor that may affect their plasticizing capacities. It is a well-known truth that low MW polar organic compounds are more effective plasticizers than high MW ones since they easily interact with embedded sites of folded biopolymers as protein and decrease their intermolecular interactions.[29] Thus, it seems that the low molecular size of LA (MW: 90 Dalton) allowed its more effective diffusion than MA (MW: 134 Dalton) and TA (MW: 150 Dalton) inside the zein structure, and this caused the formation of extensive intramolecular H-bonds with zein rods and increased the free volume of the film matrix. In contrast, it appears that the interactions of TA occurred mainly at the surface and easily accessible sites of zein. Therefore, different plasticizing effects of OAs should be related not only to the number of H-bonds they created but also to locations of H-bonding interactions and their effects on structural organizations of zein within the film matrix.

The maximum flexibility value, EB at 400%, obtained by LA at 4% in the current study is the second highest flexibility reported in the literature for zein films after maximum EB of ~530% obtained by
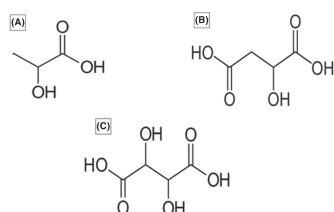
glycerol-polyethylene glycol mixture (1:2) used in films at 0.45 g/g zein.[23] The EB of 189% obtained by MA at 4.5% in the current study was comparable with those maximum EB values of 182% and 189% determined for gallic acid and hydroxybenzoic acid (each used at 3 mg/cm^2 of films) in zein films, respectively.[24] However, the EB of MA-plasticized films in the current study was slightly higher than that EBs of 145% and 165% achieved first by plasticization of zein films by addition of linoleic or oleic acid at 0.6 and 0.7 g/g zein and then replasticization of films by dipping into linoleic or oleic acid solution, respectively.[44] It is also important to note the maximum EBs of ~240% and ~350% obtained for zein films by glycerol-poly (tetramethylene) glycol mixture (1:1) and glycerol–polypropylene glycol mixture (1:2) used at 0.45 g/g zein, respectively.[23] However, although the mixtures of glycerol with different glycols or fatty acids such as oleic and linoleic acids could plasticize zein films, they cannot give antimicrobial films as OAs employed in the current work and phenolic compounds reported by Arcan and Yemenicioğlu.[24]

3.2 | Effect of storage on mechanical properties of plasticized films

The mechanical properties of zein are not stable and need to be proved by detailed storage tests since interactions among film components and water absorption or drying of films during storage affect their mechanical properties.[21,45,46] The effect of storage at 4°C and 25°C on mechanical properties was investigated only with films loaded with 3.5% LA and MA since films with TA did not show sufficient flexibilities (Figure 2). The EB and TS of control zein films stored at 4°C showed slight reductions while changes in these mechanical properties at 25°C were very limited. In contrast, films with LA and MA showed significant increases in EBs within 20 days of storage. Changes in EBs of films both with LA and MA at 4°C occurred slightly more rapidly than those at 20°C, but EBs reached separately for LA and MA within 20 days were similar for both storage temperatures. The increases in EBs of films with LA and MA stored at different temperatures were almost 1.8- to 1.9-fold (up to 450%–460%) and 2.7- to 2.9-fold (230%–242%) within 20 days, respectively. However, further storage of films for 30 days reduced the EBs of both LA- and MA-loaded films back close to their initial EBs. These results showed that dynamic changes occurred due to continued interactions between OAs and the zein film matrix. It appears that the diffusion of OAs among and within zein molecules in the film matrix improved as time progressed; thus, plasticization (increased mobility of asymmetric zein rods) continued up to the 20th day of storage. However, further development of interactions (e.g., H-bonding and Van der Waals forces) after 20 days increased film networking and caused some antiplasticizing effect due to reduced mobility of zein molecules. The changes in TSs of films with both OAs showed resemblance with this hypothesis. The TSs of LA and MA loaded films showed a significant drop at 4°C by 10-day storage, but after that, films showed a gradual increase in their TSs by 20- and 30-day cold storage. The changes in TSs at 25°C showed a different pattern with a fluctuation (increase

FIGURE 1 Molecular structures of (A) lactic acid, (B) malic acid and (C) tartaric acid
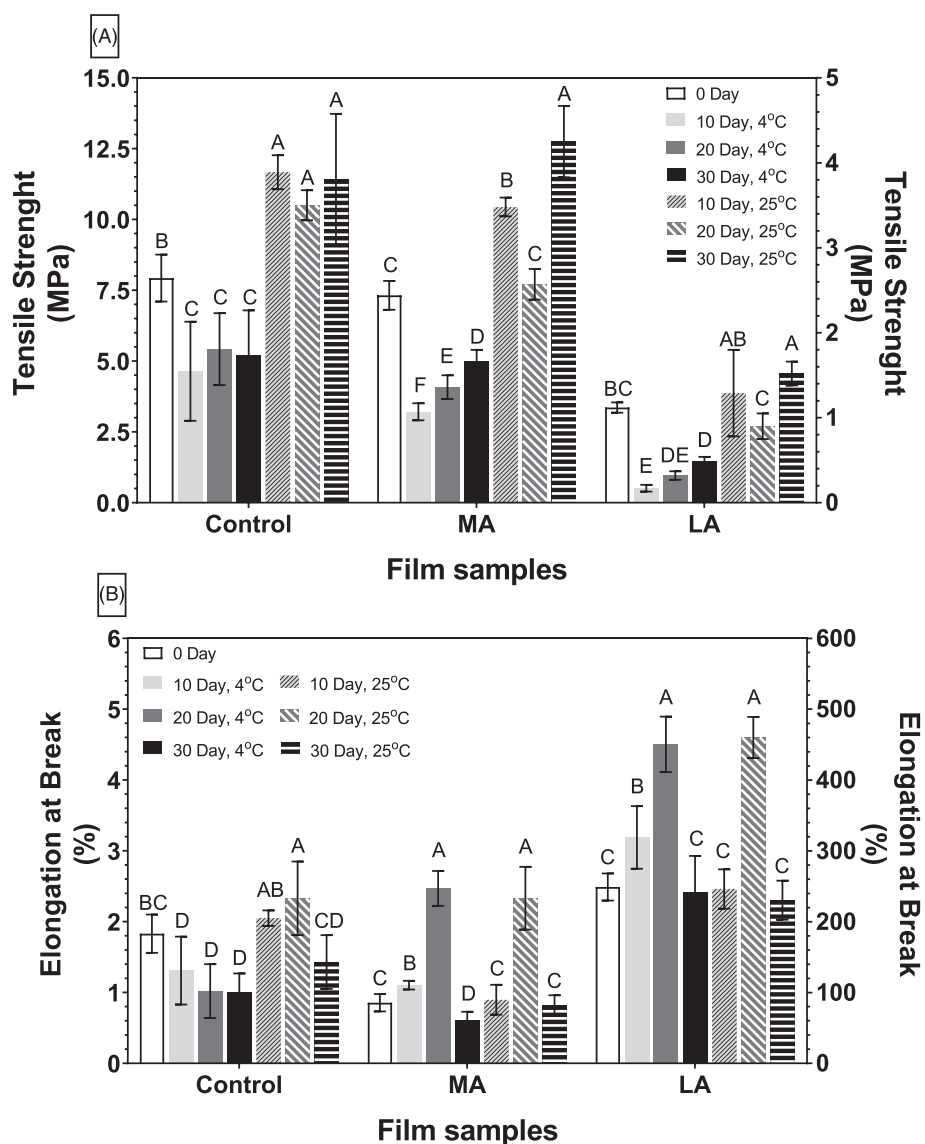
FIGURE 2 Effects of storage time (10, 20 or 30 days) and temperature (4°C or 25°C) on tensile strength (A) and elongation at break (B) of zein films plasticized with different organic acids (control films: Primary *Y*-axis, films plasticized with LA and MA: Secondary *Y*-axis). Different letters denote a statistically significant difference within each sample groups, $p \le 0.05$

and then drop) at the 10th and the 20th days followed by a significant increase on the 30th day. It is important to note that the films stored at 25°C showed significantly higher TS than those stored at 4°C. It seemed that zein in films stored at 25°C created much more intermolecular crosslinking interactions with OAs than those stored at 4°C. The higher TS of zein films stored at room temperature than those stored under refrigeration was also observed by Gennadios et al.[45] and Guo et al.[46] However, the former researcher group attributed this phenomenon to the evaporation of more water at elevated storage temperatures and the loss of this effective plasticizer while the latter group hypothesized that this phenomenon occurred due to the emerging interactions among zein molecules.

3.3 | FTIR analysis of plasticized films

Changes in functional groups, as well as bond stretching and bending, occurred within the zein film matrix by the addition of OAs

were monitored spectroscopically via FTIR (Figure 3). The main absorption peaks determined for zein film were as follows: $3,288$ cm^{-1} for amide A band due to N—H and O—H stretching; $1,651$ cm^{-1} for amide I band originating from C=O stretching vibrations; $1,546$ cm^{-1} for amide II band due to N—H bending and C—N stretching.[47] The FTIR spectra of OA loaded films were similar to the control film except for additional peaks between 1,712- and 1,730-cm^{-1} band which was attributed to carbonyl groups that came mainly from OAs while crosslinking with zein chain.[48] The position of amide A band upshifted from 3,288 to 3,292 and $3,290$ cm^{-1} by the addition of LA and MA into films, respectively (Table 2). The intensities of shifted bands for LA and MA added films were also found significantly lower than that of control film ($p \le 0.05$). This finding suggested that LA and MA caused the destruction and/or weakening of intermolecular and/or intramolecular H-bonding at N—H groups. In contrast, the addition of TA did not cause a shift in the amide A band and did not reduce the band intensity at 3,288 cm^{-1} significantly ($p > 0.05$).
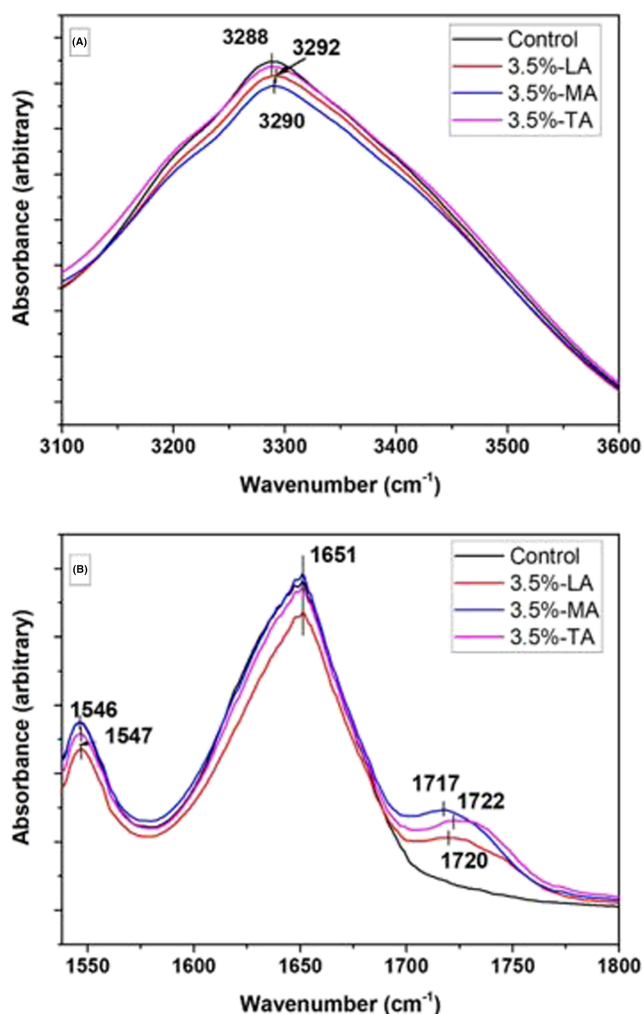
A distinct band at 1,651 cm^{-1} within the amide I region, suggested the presence of α-helix,[49] reduced significantly for both LA- and TA-loaded films while MA addition increased this band significantly ($p \leq 0.05$). However, a more detailed analysis of the amide I region is essential to understand changes in films with plasticization as many other types of secondary structure bands overlap in this region.[39] Thus, FTIR spectra were deconvoluted between 1,600 to 1,700 cm^{-1} to reveal the complex changes in different secondary structures (see Figures S1–S4). The percentages of different secondary structures determined from these bands are presented in Table 3. According to these results, the addition of all OAs increased the percentages of α-helix and random coil conformation in zein films significantly ($p \leq 0.05$). The most significant increases in these two conformations occurred with MA followed by LA and TA. Thus, analysis of α-helices both at a single band at 1,651 cm^{-1} as well as at the band range of 1,648–1,658 cm^{-1} suggested an increase in helical structures in zein films by the addition of MA. Moreover, careful analysis at the band range of 1,648–1,658 cm^{-1} instead of a single band at 1,651 cm^{-1} helped to reveal increases in α-helices of films loaded with LA and TA. The addition of LA and TA also caused limited and moderate reductions in intramolecular β-sheets and β-turns of zein in the film matrix, respectively. In contrast, the addition of MA seemed to cause dramatic deformation on intramolecular β-sheets and β-turns in the zein film matrix. LA is the only OA that caused a significant reduction in intermolecular β-sheets of zein while TA and MA caused limited and significant increases in intermolecular β-sheets of zein, respectively. The overall results of FTIR analysis indicated that increasing the amount of helical and random coil structures at the expense of β-sheet conformations is consistent with the previously determined changes in the secondary structure of zein in films by the effect of plasticizing agents such as oleic acid and glycerol.[22,50] There are also various reports in the literature that effective plasticizers

FIGURE 3 FTIR spectra of zein films plasticized with different organic acids; (A) amide a region, (B) amide I and amide II regions

TABLE 2 FTIR absorption intensities and peak wavenumber of different zein films

Organic acid	Peak wavenumber (cm^{-1})	Intensity (abs)	Peak wavenumber (cm^{-1})	Intensity (abs)	Peak wavenumber (cm^{-1})	Intensity (abs)
Control	3,288	1.50 ± 0.02^A	1,651	2.40 ± 0.01^B	1,546	1.37 ± 0.02^A
3.5% LA	3,292	1.43 ± 0.01^B	1,651	2.17 ± 0.01^D	1,547	1.18 ± 0.01^C
3.5% MA	3,290	1.39 ± 0.01^C	1,651	2.45 ± 0.01^A	1,546	1.37 ± 0.01^A
3.5% TA	3,288	1.47 ± 0.02^A	1,651	2.36 ± 0.01^C	1,547	1.29 ± 0.01^B

Note. Values at each column indicated by different letters are significantly different ($p \leq 0.05$).

TABLE 3 FTIR amide I deconvolution areas (%) of zein films prepared with organic acids

| Organic acid (3.50%) | Intermolecular β-sheet | | Intramolecular β-sheet | | β-Turn | Random coil | α-Helix |
	1,610–1,625 cm^{-1}	1,685–1,695 cm^{-1}	1,670–1,684 m^{-1}	1,630–1,640 cm^{-1}	1,660–1,668 cm^{-1}	1,640–1,648 cm^{-1}	1,648–1,659 cm^{-1}
Control	14.0 ± 0.05^C	0.96 ± 0.02	10.7 ± 0.06^A	23.5 ± 0.12^A	13.6 ± 0.05^A	15.5 ± 0.05^D	21.7 ± 0.10^D
LA	12.8 ± 0.21^D	–	10.9 ± 0.16^A	22.2 ± 0.03^C	13.7 ± 0.13^A	16.6 ± 0.12^B	23.8 ± 0.13^B
MA	17.2 ± 0.14^A	–	8.84 ± 0.07^C	20.8 ± 0.12^D	12.3 ± 0.05^C	16.8 ± 0.04^A	24.2 ± 0.05^A
TA	14.4 ± 0.16^B	–	9.74 ± 0.09^B	23.2 ± 0.08^B	12.8 ± 0.10^B	16.2 ± 0.03^C	23.6 ± 0.07^C

Note. Values at each column indicated by different letters are significantly different ($p \leq 0.05$).

interfere with embedding interactions and H-bonding among interior parts of proteins in the film matrix while favouring the formation of helical structures and random coils.[51,52] However, Huo et al.[23] reported that plasticization of zein film by a combination of plasticizers glycerol and polyethylene glycol could be related to the disruption of inter-helical packing and increased β-sheet content. Moreover, they also hypothesized that the plasticizers interfered between packed β-sheet and prevented their interaction via hydrogen bonding. Thus, it is possible that the specific impact of effective plasticizer LA originates partly from spacing among intermolecular β-sheets while that of MA occurs by the contribution of increased helical structures as well as spacing among intramolecular β-sheets. It also appears that the ineffectiveness of TA to plasticize zein films is due to its high MW that limits its diffusion into depths of zein structure and prevents its dramatic effects on secondary structures.

The amide II band region originating mainly from N—H bending vibrations is sensitive to differences in hydrogen bonding.[53] The results showed that the addition of OAs did not cause a considerable amide II band shifting. In contrast, significant decreases were observed in intensities at 1,547 cm^{-1} for LA and TA loaded films while films with MA did not affect the intensity of the amide II band. The results for LA- and TA-loaded films supported the findings of Gillgren et al.[54] and Gao et al.,[50] who also detected a reduction in amide II band intensity for zein films after they were plasticized by the addition of glycerol, water or 4-mercaptoethanol, and glycerol, respectively. These authors attributed the reduction in amide II band intensity of zein films to increased plasticizer/amide interaction due to H-bonding at the expense of amine/amide interaction. However, these analyses of amide II band vibrations at 1,546 cm^{-1} cannot explain the

lack of any changes in the current study in band intensity of films loaded with MA that possibly induced some conformational changes (e.g., raised intensities) that balance reduced amide II band intensities.

3.4 | Morphological characteristics of films

The cross-sectional morphologies of control and OA-loaded zein films monitored through SEM are displayed in Figure 4A–D. As seen in Figure 4A, control films had a porous structure due to the evaporation of ethanol during film drying as described previously.[55,56] The incorporation of LA did not cause a remarkable change in film morphology (Figure 4B) while the incorporation of MA (Figure 4C) and TA (Figure 4D) caused some slight to moderate and extensive modifications in zein film morphology, respectively. It appeared that the changes in MA-loaded films are limited with heterogeneous structural changes, possibly caused by zein protein aggregates, at areas close to the film surface and slightly below these surface areas (see black arrows). Some rare local nonhomogeneous aggregates were also observed distributed within the central parts of films (see white arrows). In contrast, dramatic changes in morphologies of TA-loaded films occurred such as the formation of porous layers at the surface and bottom of films with an interfering dense layer (see explanations in Figure 4D). It appears that TA-loaded films underwent some phase separation during drying that caused first the formation of a dense layer at the upper part and a porous layer at the bottom of films, and then the formation of a second thinner, porous layer at the top by continued evaporation of ethanol. It seems that the dense layer was formed due to the concentration of TA in the upper phase during the
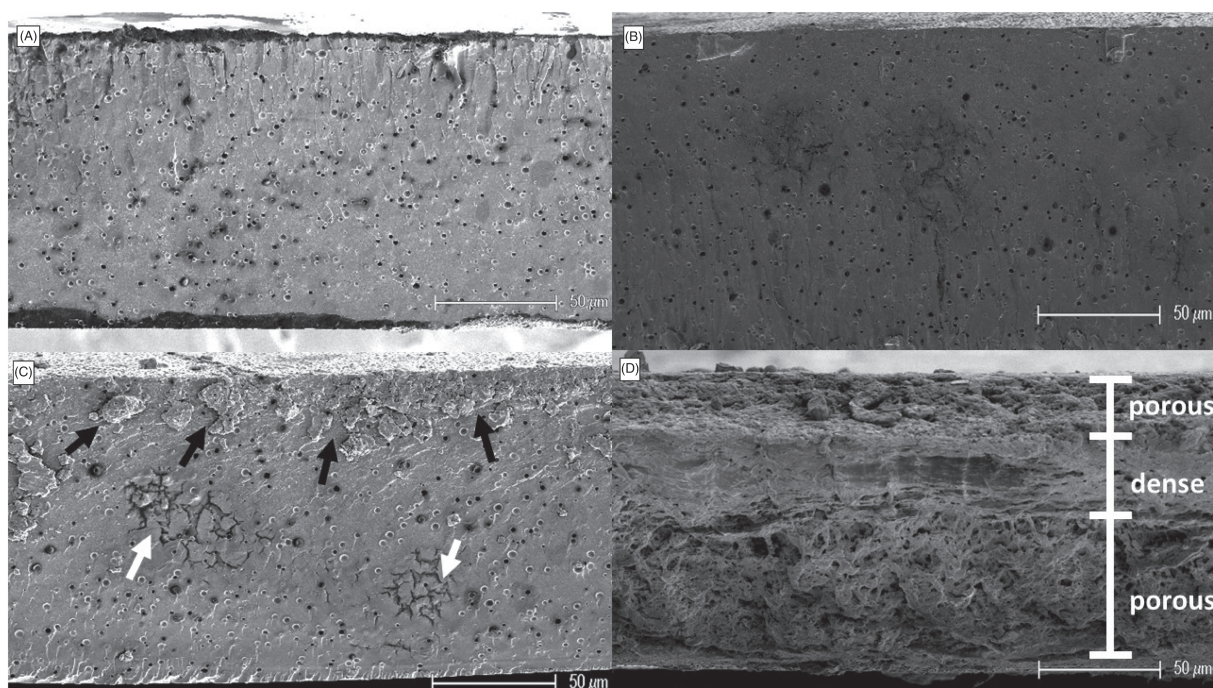
FIGURE 4 Effect of organic acids on the cross-sectional morphology of different zein films: (control film: (A) films with 3.5% LA: (B) 3.5% MA: (C) 3.5% TA: (D) magnifications: 500×)

evaporation of ethanol and following intensified intermolecular changes (crosslinking and polymerization) in zein at the upper phase of films. These observations clearly proved that LA gave the most homogenous film morphology.

The surface morphologies (Figure 5A–D), topographic images (Figure 6A–D) and roughness parameters (R_{rms} and R_{max}) (Table 4) of OA incorporated zein films were obtained through AFM. Figures 5A and 6A show that control zein film had large and deep pores with significantly higher roughness parameters than OA loaded films ($p \leq 0.05$). In contrast, LA-loaded (Figure 5B and 6B) and MA-loaded (Figure 5C and 6C) zein films showed similar, but significantly less rough surfaces than control films and TA-loaded films. The morphological features of TA-loaded zein film are also provided in Figures 5D and 6D. The roughness parameters of TA-loaded films changed between those of controls and other OA-loaded zein films. Thus, it is clear that all OAs reduced the roughness and pore depth of zein films, but LA and MA were much more effective than TA to improve the classical roughness problems associated with zein films.

3.5 | Effect of different OAs on the WVP of films

The WVPs of OA-loaded zein films were shown in Figure 7. The highest WVP was determined for LA loaded zein films followed by those of control zein film, and MA and TA loaded films. The film WVP is related to different factors such as the hydrophilic or hydrophobic properties of the film-forming polymer, the degree of film crosslinking and the presence of voids, cracks, tortuosity or steric hindrance effects in the film matrix.[57,58] Although the zein is mainly a hydrophobic protein, the weak chain association and voids present in its structure might have contributed to the high WVP of control films as indicated previously by Ghanbarzadeh et al.[59] In general, plasticization of edible films including zein causes an increase in their WVP since binding of plasticizer onto polymer molecules' surface increases their chain spacing and mobility.[57,60,61] The significant increase in WVP of LA-loaded films shows similarity with this generally accepted rule since these films were plasticized effectively. In contrast, the reduced WVP of TA-loaded films might be related to the lack of TA's ability to increase plasticity (intermolecular spacing) in zein film while causing increased crosslinking of the film matrix via H bonding. Moreover, the dense intermediate layer observed in TA-loaded films should also be a factor reducing the WVP of films (see Figure 4D). Furthermore, it seems that the reduced WVP by MA that also showed a considerable plasticizing effect was related to complex secondary structural changes caused by this OA. The FTIR results in the current work suggested that LA is the only OA that caused a significant reduction in intermolecular β-sheets of zein. However, the FTIR results also suggested that MA caused
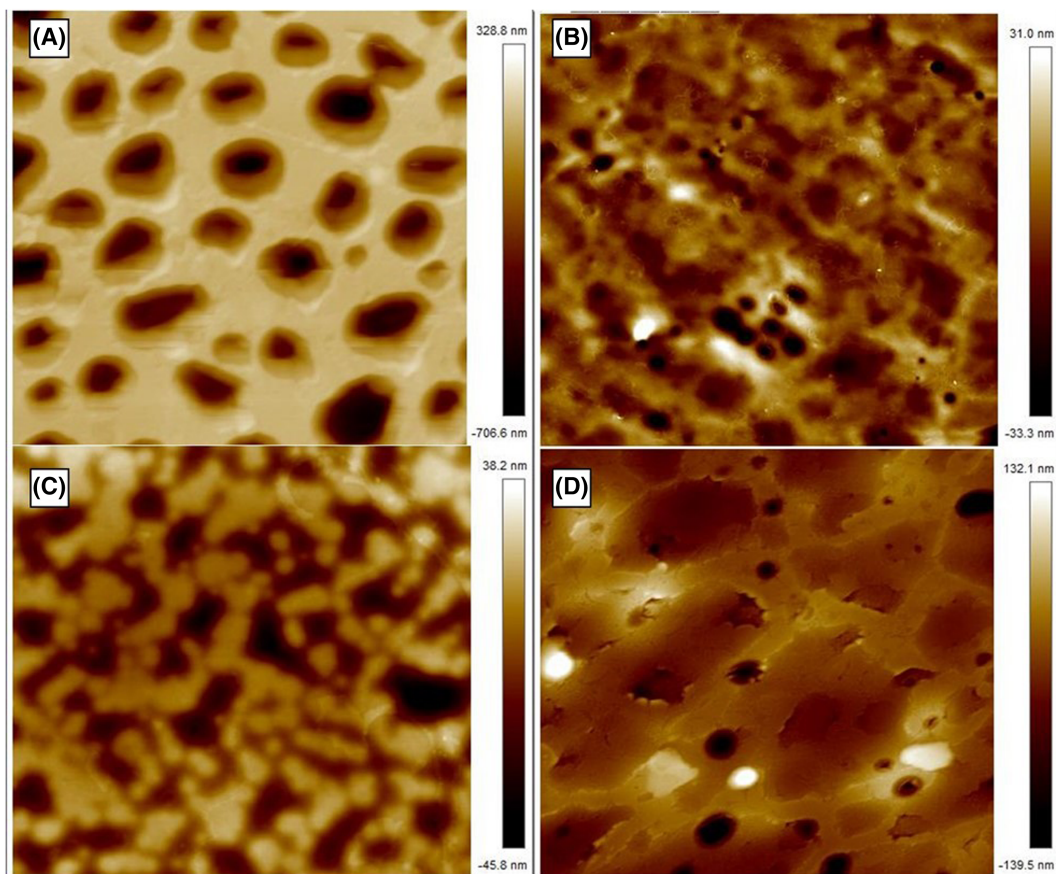
FIGURE 5 Atomic force microscopy height images ($10 \times 10 \ \mu m^2$) of different zein films (control film: (A) films with 3.5% LA: (B) 3.5% MA: (C) 3.5% TA: (D))

(A)

(B)

(C)

(D)
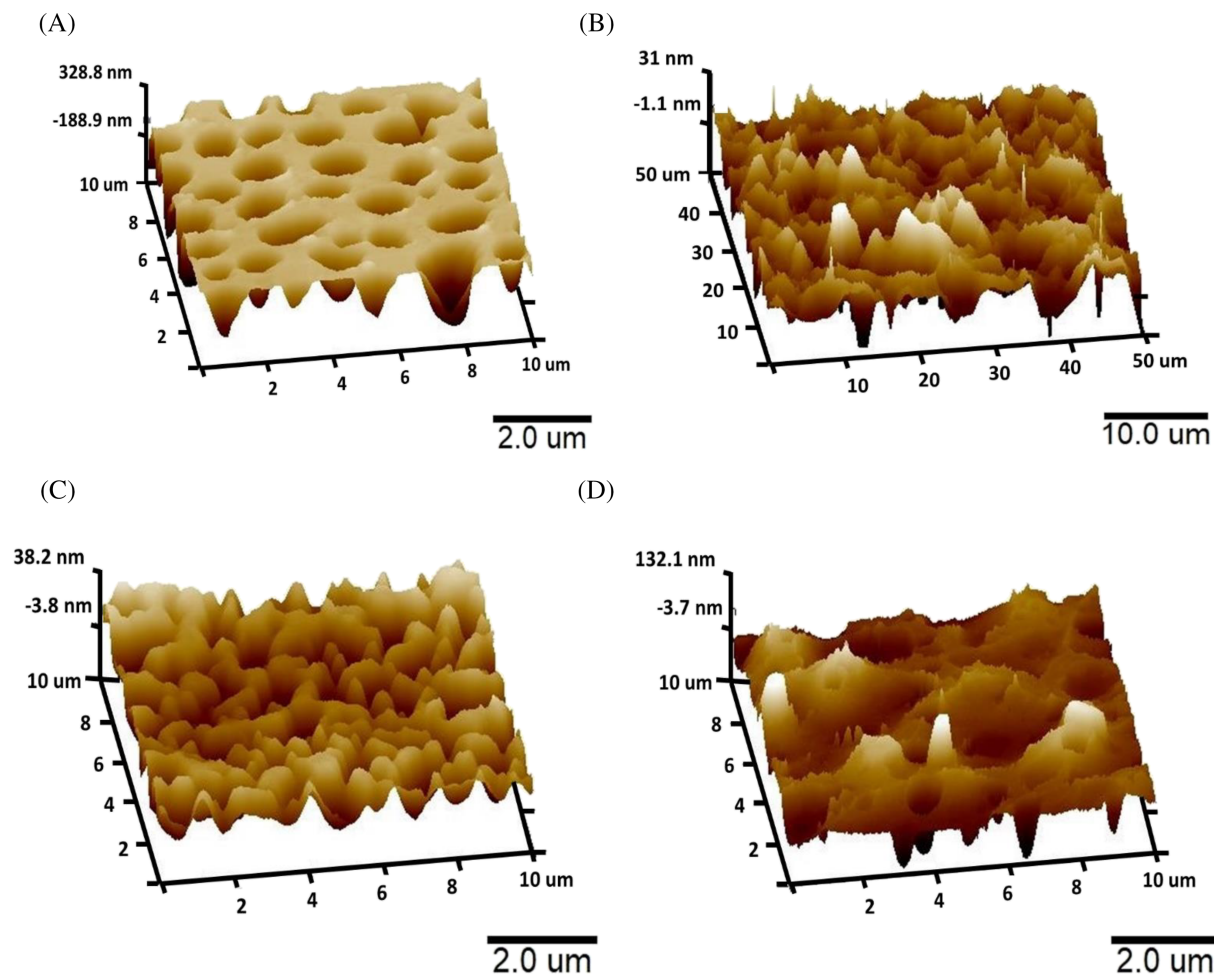
FIGURE 6   Topographic images of different zein films (control film: (A) films with 3.5% LA: (B) 3.5% MA: (C) 3.5% TA: (D))
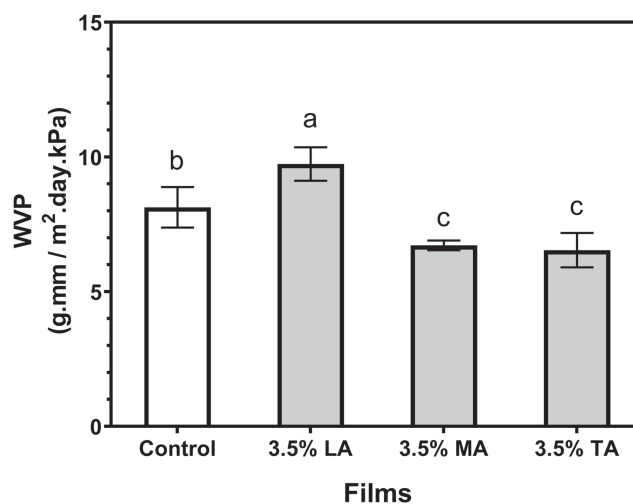
TABLE 4   Morphological parameters of different films from AFM analysis

Organic acid	R_{rms} (nm)[a]	R_{max} (nm)
Control	69.4 ± 14.4[A]	443 ± 62.1[A]
3.50% LA	3.02 ± 1.76[C]	42.4 ± 36.9[C]
3.50% MA	4.52 ± 1.75[C]	34.3 ± 10.3[C]
3.50% TA	20.0 ± 11.8[B]	203 ± 112[B]

[a]Values at each column indicated by different letters are significantly different ($p ≤ 0.05$).

FIGURE 7   WVP of zein films plasticized with different organic acids (different letters denote a statistically significant difference among samples, $p ≤ 0.05$)

dramatic deformation on intramolecular β-sheets and β-turns in the zein film matrix while it had caused significant increases in intermolecular β-sheets of zein. The β-sheets are known with their hydrophobic nature that is a critical factor effective on film WVP.[62] Thus, it appears that the sharp differences in number and positions of β-sheets are a major factor affecting the dramatic difference between WVP of films plasticized with LA and MA.

In the literature, results of WVP for plasticized zein films showed some variations due to differences in film composition and test conditions. However, analysis of different zein films gives information about possible ranges of WVP for these films. For example,

Ghanbarzadeh et al.[59] reported that the WVP (at 15% RH) of unplasticized zein film was 46.2 g mm m² day kPa whereas the WVP of zein films plasticized with glucose, galactose and fructose

(each at 0.5, 0.7 or 1 g/g of zein) ranged at 33.7–40.7, 27.6–31.8 and 51.7–57.7 g mm m^2 day kPa, respectively. Huo et al.[23] reported that WVP (at 97% RH) of glycerol plasticized control zein films ranged between 64.8 and 76.8 g mm m^2 day kPa while films plasticized with poly(propylene glycol) (PPG), poly(tetramethylene glycol) (PTMG) or poly(ethylene glycol) (PEG) (each at 0.35-, 0.40- or 0.45-g plasticizer/g zein) ranged between 16.8 and 28.8 g mm m^2 day kPa. According to Parris and Coffin,[63] WVP (at 0% RH) of control zein films and zein films plasticized with glycerol:polypropylene glycol (MW 400, at 1:3 ratio and 30%, w/w in film-forming solution) changed between 14.9 and 25.4 g mm m^2 day kPa. It is evident that the WVP values of both control and OA plasticized zein films (changed between 6 and 10 g mm m^2 day kPa) in the current study were considerably lower than those reported above for different plasticized zein films in the literature. However, the WVPs reported in the current work were only slightly higher than those reported (1.7–3.5 g mm m^2 day kPa at 90% RH) by Yoshino et al.[64] for unplasticized zein films produced at different drying temperatures (35°C or 45°C) and RH (5% or 90% RH). It should also be noted that the WVPs of films developed in the current study were comparable with that of whey protein isolate films (7.7 g mm m^2 day kPa at 25% RH) by Oymaci and Altinkaya[65] but they were higher than that reported by Manab et al.[66] for whey protein films incorporated with LA (0.28 g mm m^2 day kPa at 0% RH for 5%, v/v, of LA). However, zein films are much hydrophobic than whey protein films; thus, they show less wettability and swelling during flexible packaging applications.

3.6 | Antimicrobial activity of films based on disc-diffusion method

The antimicrobial tests were conducted only with LA and MA that gave the desired plasticization effect. The results of antimicrobial tests for 3%, 3.5% and 4% LA- and MA-loaded films against *L. innocua*, *E. coli*, *S. aureus* and *K. pneumoniae* are presented in Table 5. Films loaded with 3%, 3.5% and 4% LA or MA were effective on *L. innocua* and *K. pneumoniae* since all discs tested from these films formed clear zones on these bacteria. Zones formed against *L. innocua* by both OA-loaded films were significantly larger (3- to 10-fold) than those formed by films against *K. pneumoniae* (*p* ≤ 0.05). In fact, it is important to note that *L. innocua* was the most susceptible bacteria against OAs tested. Films with MA formed significantly larger zones against *L. innocua* than films with LA, but LA-loaded films performed better than MA-loaded films against *K. pneumoniae* (*p* ≤ 0.05). The most resistant bacteria against OAs were *S. aureus*. Therefore, films with OAs did not form clear zones against this Gram-positive bacterium. Instead, extensive regrowth of colonies was observed in zone areas around discs. However, lack of microbial growth under the LA- and MA-loaded discs indicates a very limited antimicrobial effect of these OAs against *S. aureus*. *E. coli* also showed some resistance against MA-loaded films and formed unclear zones at all concentrations. In contrast, LA-loaded films formed clear fully formed zones against *E. coli* except for concentration at 3%.

In literature, studies related to the antimicrobial activity of edible zein films loaded with OAs such as LA and MA are scarce.

TABLE 5 Zone inhibition based antimicrobial activities of organic acid loaded zein films

Test bacteria	Conc. (%) w/w	Lactic acid Zone area^a (cm^2)^b	Malic acid Zone area (cm^2)
L. innocua	0	NZ	NZ
	3.00	7.03 ± 0.71^{b,B}	9.00 ± 0.97^{a,A}
	3.50	6.91 ± 0.86^{b,B}	9.72 ± 1.32^{a,A}
	4.00	8.02 ± 0.79^{b,A}	9.47 ± 0.62^{a,A}
S. aureus	0	NZ	NZ
	3.00	UCZ-LA	UCZ-LA
	3.50	UCZ-LA	UCZ-LA
	4.00	UCZ-LA	UCZ-LA
E. coli	0	NZ	NZ
	3.00	UCZ-LA	UCZ-LA
	3.50	1.33 ± 0.15^{a,CD}	UCZ-LA
	4.00	1.55 ± 0.67^{a,CD}	UCZ-LA
K. pneumoniae	0	NZ	NZ
	3.00	0.80 ± 0.20^{a,D}	0.94 ± 0.31^{a,B}
	3.50	1.32 ± 0.35^{a,CD}	0.61 ± 0.12^{b,B}
	4.00	2.15 ± 0.65^{a,C}	1.27 ± 0.38^{b,B}

^{a}Values at each column and raw followed by different capital (A–D) and initial (a and b) letters indicate statistically significant differences (*p* ≤ 0.05), respectively.

Abbreviations: LA, limited antimicrobial effect (no microbial growth under the disc); NZ, no zone; UCZ: unclear zone.

TABLE 6 Surface inoculation based antimicrobial activity of organic acid loaded zein films

Organic acid in film	Concentration (%) (w/w)	Storage time (days)	E. coli (log CFU g^{-1})[a]	S. aureus (log CFU g^{-1})
Control	—	0	6.63 ± 0.20[A,b]	7.62 ± 0.24[A,a]
LA	3.50	0	<2.3	6.25 ± 0.10 [C]
MA	3.50	0	<2.3	5.24 ± 0.27 [D]
Control	s	1	6.40 ± 0.14[A,b]	7.52 ± 0.12[AB,a]
LA	3.50	1	<2.3	<2.3
MA	3.50	1	<2.3	<2.3
Control	—	7	3.57 ± 0.19[B,b]	7.32 ± 0.12[B,a]
LA	3.50	7	<2.3	<2.3
MA	3.50	7	<2.3	<2.3

[a]Values at each column and raw followed by different capital (A–D) and initial (a and b) letters indicate statistically significant differences ($p \leq 0.05$), respectively.

However, Eswaranandam et al.[67] tested soy protein films with different OAs and determined that MA was a more potent antimicrobial than LA against *L. monocytogenes*, *but films with both of these OAs showed* similar antimicrobial activity against *Salmonella gaminara* and *E. coli* O157: H7. Zhong et al.[68] who prepared kudzu starch–chitosan composite films with MA or LA, reported that MA showed better antimicrobial activity than LA against *E. coli* and *S. aureus*. Pintado et al.[69] also reported the higher antimicrobial effect of 3% MA-loaded whey protein films than 3% LA-added films against *L. monocytogenes*. Our results related to higher anti-listerial activity of MA than LA compared well with the literature. However, the findings of current work about the superior antimicrobial performance of LA against *E. coli* contradicted the findings of Zhong et al.[68] The variations in the antimicrobial performance of different types of edible films loaded with similar OAs could be related to differences in bound/soluble OA ratios of hydrophilic and hydrophobic films. Moreover, the variations in abilities of OAs to penetrate microbial cell walls and membranes, to create acidity (pKa) as well as variations in microbial strains and test conditions could also be effective on different results reported. The pKa of monocarboxylic acid LA is 3.86 while dicarboxylic acid MA showed pKa values of 3.4 and 5.11 for its carboxyl groups.[70] Thus, it is clear that MA shows a higher capacity to produce acidity at physiological pH close to neutrality. However, the ability of OAs to interact with bacterial cell walls varies considerably. The LA has a lower MW than MA as mentioned in Section 3.1. Moreover, it was reported that LA could destabilize lipopolysaccharides at the cell walls of Gram-negative bacteria like *Salmonella enterica* serovar typhimurium much more effectively than HCl and ethylenediaminetetraacetic acid (EDTA).[71] In contrast, Rathnayaka[72] reported that *E. coli* was resistant against MA due to the low ability of this OA to penetrate the lipopolysaccharide layer of this Gram-negative bacterium. This report supported our findings that indicated the superior antimicrobial effect of LA-loaded films against *E. coli* than those loaded with MA.

3.7 | Antimicrobial activity of films based on surface inoculation method

The surface inoculation tests were conducted with *E. coli* and *S. aureus* since these bacteria showed resistance against LA- and MA-loaded films in disc-diffusion tests (Table 6). The inoculation and storage tests of control films for 7 days clearly showed the high stability (only 0.3 log reduction) of *S. aureus* on the hydrophobic zein film surface. The *E. coli* inoculated at the control zein film surface was stable for 1 day, but it showed almost 3-log reduction within 7-day storage. This finding that suggested the instability of *E. coli* on hydrophobic zein surface showed parallelism with recent findings of Boyacı et al.[26] The test of LA- and MA-loaded films against bacteria clearly showed the antimicrobial potential of these films. For both LA- and MA-loaded zein films, the *E. coli* showed a minimum 4.3-log reduction at time 0, and no bacterial regrowth was observed on films within 1 week. In contrast, *S. aureus* showed more resistance against LA and MA and showed almost 1.4- and 2.4-log reduction on the surface of these films at time 0, respectively. These results also suggested a greater antibacterial effect of MA- than LA-loaded films on *S. aureus* ($p \leq 0.05$). However, *S. aureus* loads of all OA-loaded films drop below 2.3 log CFU g^{-1} within 1-day storage, and they remained at this level for 7 days at 10°C. Thus, the overall results clearly showed that the *E. coli* and *S. aureus* resisted against released LA and MA from films in disc-diffusion tests did not survive at OA-loaded film contact surfaces.

4 | CONCLUSIONS

This work proved the good potential of using OAs like LA and MA to increase flexibility and antimicrobial activity of zein films that could not have found a widespread application due to their well-known brittle nature. The flexibility obtained using LA is one of the highest among the zein plasticization studies. Moreover, the retention of

gained flexibility of films up to 1 month both at room temperature and refrigerated storage clearly showed the applicability of films as an antimicrobial coating in a wide range of food products. The films loaded with LA and MA showed antimicrobial activity on critical food pathogenic bacteria. The LA is a much more effective plasticizer than MA, but the MA gives films with considerably lower WVP than LA. Thus, the findings of this work may provide an alternative opportunity to initiate flexible antimicrobial film applications of corn zein a very important by-product of rapidly growing bioethanol and oil industries.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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