Design and Characterization of Corn Starch Edible Films Including Beeswax and Natural Antimicrobials

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Abstract

The effectiveness of edible films (EFs) used as coatings to maintain the quality and safety of fresh produce for long time depends on their functional properties characterization. This study was aimed to design and evaluate physicochemical, barrier, mechanical, and antimicrobial properties of EFs based on corn starch

(acetylated cross-linked (ACLS) or oxidized (OS)), micro-emulsified beeswax (BW, 0–1 % *w*/*w*), and two natural antimicrobials (lauric arginate (LAE, 400–4000 mg/L) and natamycin (NAT, 80–800 mg/L)). EFs based on ACLS or OS made with 1 % BW microemulsion produced homogeneous EFs surface and did not show changes in thickness or opacity. Water vapor permeability (WVP, 0.57 ± 0.04 g mm m^{-2} h^{-1} kPa^{-1} for ACLS, and 0.56 ± 0.05 g mm m^{-2} h^{-1} kPa^{-1} for OS) was reduced; tensile strength (TS, 51.48 ± 5.92 MPa for ACLS, and 40.96 ± 4.98 MPa for OS), and elastic modulus (EM, 211.30 ± 7.85 MPa for ACLS, and 203.50 ± 5.35 MPa for OS) were decreased, whereas elongation at break (E, 4.59 ± 1.11 % for ACLS, and 4.76 ± 4.98 % for OS) increased. The additive effect showed by the combination of natural antimicrobials (2000 mg/L of LAE plus 400 mg/L of NAT) incorporated into EFs with 1 % BW completely inhibited *Rhizopus stolonifer*, *Colletotrichum gloeosporioides*, *Botrytis cinerea*, and *Salmonella* Saintpaul. These properties of corn starch EFs used as coatings represent an excellent alternative to extend the shelf life of fresh produce.

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Keywords

Edible films
Modified corn starch
Micro-emulsified beeswax
Antimicrobials

Abbreviations

ACLS	Acetylated cross-linked starch
BW	Beeswax
E	Elongation at break
EFs	Edible films
EM	Elastic modulus
LAE	Lauric arginate
NAT	Natamycin
OS	Oxidized starch
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RBA	Rose bengal agar
RH	Relative humidity

TS	Tensile strength
TSA	Tryptone soy agar
TSB	Tryptone soy broth
WVP	Water vapor permeability

Introduction

Extensive use of synthetic packaging has caused serious environmental problems due to waste generation of low degradability solids (Lin and Zhao 2007; Olivas and Barbosa-Cánovas 2009). The design of edible films (EFs) is becoming more and more relevant in food preservation because they offer selective functionality such as regulation of moisture transfer, selective gas barrier, retention of flavor components, improvement of mechanical properties, and also serve as additives vehicle (antimicrobials, antioxidants, etc.)(Janjarasskul and Krochta 2010). In addition, EFs are sensory acceptable and can be produced from natural and biodegradable polymers (Jiménez et al. 2012). EFs based on proteins and polysaccharides suffer from poor water vapor permeability (WVP), whereas those based on lipids are opaque and sensory unstable (Olivas and Barbo~~z~~sa-Canovas~~,~~ 2009; Soliva-Fortuny et al. 2012). Some properties of EFs are still in the research stage, such as demonstration of effectiveness, food safety issues, and cost (Dhall, 2013).

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Starch is a renewable polysaccharide commonly used in the food packaging area due to its abundance, low cost, and commercial availability. Native corn starch easily gelatinizes and is capable of forming EFs by thermoplastic processing, but low shear resistance, thermal decomposition, and high tendency to exhibit retrogradation limit its use in some industrial food applications. However, chemical modifications involving the introduction of functional groups can improve starch functionality through reactions such as esterification, cross-linking, or oxidation. EFs based on modified corn starch are clear, flexible, and provide selective barrier to CO_2 and O_2, but show a poor water vapor barrier (Singh et al. 2007; Jiménez et al. 2012).

Usually, the addition of hydrophobic agents such as beeswax (BW) to the EFs formulation has led to improved moisture barrier and mechanical properties, and appearance of fresh produce when applied as coatings (Han et al. 2006; Navarro-Tarazaga et al. 2008). Natural antimicrobials such as lauric arginate (LAE) possess

a broad spectrum against bacteria (Gram-positive and Gram-negative), fungi, and yeasts, whereas natamycin (NAT) is used to control fungal and yeast growth (Delves-Broughton et al. 2005; Valencia-Chamorro et al. 2011; Higueras et al. 2013). Fresh produce are commonly deteriorated by *Botrytis cinerea*, *Rhizopus stolonifer*, and *Colletotrichum gloeosporioides*, resulting in ≥50 % of postharvest losses during transportation, storage, or marketing. Control of these spoilage fungi is achieved by fungicides application, but resistance and toxic residues are major disadvantages (Antunes and Cavaco 2010). *Salmonella* Saintpaul is a Gram-negative bacterium causing spoilage in fresh produce that has been involved in some outbreaks leading to high economic losses (~~CDC~~CDC 2015 ~~2016~~). There are few studies on the development of starch-based EFs using a combination of natural antimicrobials (Basch et al. 2013; Mei et al. 2013), and micro-emulsified BW as hydrophobic agent (Perez-Gago et al., 2002; Santos et al. 2014), which might be used to protect foods surface from fungal and bacterial spoilage. Thus, this study was aimed to design and evaluate physicochemical, water vapor permeability (WVP), mechanical and antimicrobial properties of EFs based on acetylated cross-linked starch (ACLS) and oxidized starch (OS), incorporating micro-emulsified BW and a combination of LAE and NAT to inhibit *B. cinerea*, *R. stolonifer*, *C. gloeosporioides*, and the pathogenic bacteria *Salmonella* Saintpaul.

Materials and Methods

Chemicals

Two commercial modified corn starches, ACLS and OS, and non-crystallizable sorbitol were provided by Ingredion (San Juan del Río, Qro., México). Tween 80 (J.T. Baker, Center Valley, PA, USA), BW, stearic acid, and morpholine were supplied by Sigma (St. Louis, MO, USA). LAE was acquired from Vedeqsa-Lamirsa (Terrassa, Barcelona, Spain) while NAT was purchased from EcoBio (Columbus, OH, USA).

Microorganisms

B. cinerea (CDBB-H-1556) and *R. stolonifer* (CDBB-H-318) were provided by the national collection of microbial strains and cell cultures (CINVESTAV, IPN, México), whereas *C. gloeosporioides* strain was ATCC 42374. *Salmonella* Saintpaul S70 was supplied from the culture collection of the center for food research and development (CIAD, CONACYT, México).

Culture Media

Potato dextrose broth (PDB) and potato dextrose agar (PDA) (Bioxon, Cuatitlán Izcalli, México), and rose bengal agar (RBA) (Difco, Sparks, MD, USA), supplemented with 0.05 % *w*/*v* chloramphenicol (Sigma), were used to grow *B. cinerea*, *R. stolonifer*, and *C. gloeosporioides*. Tryptone soy broth (TSB) and tryptone soy agar (TSA) (Bioxon) were used for *S.* Saintpaul growth.

Characterization of Starches

The two modified corn starches, ACLS and OS, were characterized. The amylose content was determined using an amylose/amylopectin kit (Megazyme, Wicklow, Ireland). OS carbonyl and carboxyl groups (Kuakpetoon and Wong, 2006) and ACLS acetylation (Sánchez-Rivera et al. 2010) were evaluated titrimetrically. The degree of cross-linking of ACLS was previously reported (Pérez-Gallardo et al. 2012). Acetylation was expressed as degree of substitution (DS), defined as the average number of hydroxyl groups per glucose unit that possess an acetyl group in the starch structure.

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Preparation of BW Microemulsion

The BW microemulsion was obtained before being incorporated into the film-forming dispersion according to Hagenmaier and Baker (1994). A mixture of molten wax (60 ± 5 °C, 28.5 % *w*/*w*), stearic acid (4.2 % *w*/*w*), and morpholine (4.2 % *w*/*w*) were added to hot water at 95 ± 5 °C, followed by high-speed homogenization at 21,500 rpm for 3 min (IKA T25-Ultra-Turrax, Wilmington, DE, USA) fitted with a S-25N-25F tip. Trying to produce a more homogeneous and stable BW dispersion, the microemulsion was subjected to 500 W ultrasonic processing using a 3-mm diameter probe (Vibra-Cell VCX 500, Newtown, CT, USA). Two 60-s pulses with resting period of 60 s, at 20 kHz frequency, and 40 % amplitude were applied in an ice bath (Kentish et al. 2008), and maximum the temperature reached was 28 ± 3 °C.

Droplet Size Determination of BW Microemulsion

Particle size of the BW microemulsions was obtained by laser light scattering using a Mastersizer 2000 coupled with a Hydro 2000S dispersion unit (Malvern Instruments, Malvern, Worcestershire, UK). The relative refractive index of the lipid phase to water was set at 1.38, and the absorption was set at 1.0. The droplet size was expressed as the Sauter mean diameter *D* (3, 2) which represents the

volume-surface average diameter associated to particles with small surfaces (Jafari et al. 2006).

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Film Preparation

Corn starch EFs were prepared by an aqueous dispersion of ACLS (3 % *w*/*w*) or OS (6 % *w*/*w*) and non-crystallizable sorbitol (0.8 g/100 g of starch) as plasticizer under continuous stirring at room temperature (25 ± 2 °C). The dispersion was heated to 90 ± 5 °C for 20 min with continuous mixing and cooled in an ice bath. Tween 80 (0.15 % *w*/*w*) and BW microemulsion at three different concentrations (0.2, 0.6, and 1 %, *w*/*w*) were incorporated into the dispersion, and then subjected to high-speed homogenization at 21,500 rpm for 3 min, followed by degassing under vacuum for 10 min. EFs were obtained by pouring 8 mL of film-forming dispersion into leveled square glass plates (10 cm × 10 cm) and allowed to dry for 24 h at constant temperature (25 ± 1 °C) and relative humidity (50 ± 1 % relative humidity, RH) chamber (Binder KBWF 240, Ösch Tuttlingen, Germany). Physicochemical, water vapor permeability, and mechanical properties of EFs were evaluated; films prepared without BW microemulsion were used as control.

Physicochemical Properties of Films

Thickness

The EFs' thickness was measured using a digital micrometer (Mitutoyo MDC-Lite, Naucalpan, México). The final value represents the average of five randomly selected points of each film (Longares et al. 2004).

Opacity

The EFs opacity was determined following López et al. (2008) and Chen et al. (2009). A film sample (2.3 cm × 0.5 cm) was placed inside a spectrophotometer cell and absorbance scan was conducted in the range 400 to 700 nm, using a UV-Vis spectrophotometer (Lambda 40, Perkin-Elmer, Waltham, MA, USA). Absorbance units per nanometer (AU × nm) were obtained by calculating the area under the absorbance versus wavelength curve.

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Water Solubility

Films rectangles (4 cm × 3 cm) were dried to constant weight at 60 ± 1 °C, and placed in glass vials containing 80 mL distilled water for 30 min at room

temperature with gentle agitation. The solution was filtered through previously dried Whatman No. 4 filter paper (GE-Healthcare, Little Chalfont, Buckinghamshire, UK); undissolved film was brought to constant weight at the same conditions. Results were expressed as percentage of soluble matter (%SM), calculated using Eq. (1) (López et al. 2008; Zamudio-Flores et al. 2009).

$$\%\mathrm{SM} = [(\text{initial dry weight} - \text{final dry weight}) / \text{initial dry weight}] \times 100 \quad (1)$$

Water Vapor Permeability (WVP) of Films

WVP was performed according to the modified standard gravimetric method ASTM E96-00 (2000). Each film was sealed between two Teflon rings with an exposed area of 9.07 cm^2 in a permeation cell with distilled water inside (100 % RH) and stored at 25 ± 2 °C in a desiccator, maintained at 44 ± 2 % RH with a saturated solution of potassium carbonate (K_2CO_3) to provide a RH gradient. Twelve weight measurements were taken during a 24-h period, plotted as a function of time, and WVP was expressed as g mm/(m^2 h kPa).

Mechanical Properties of Films

EFs strips (15 cm × 0.8 cm) were previously conditioned at 25 ± 2 °C and 44 ± 2 % RH for 24 h. A texture analyzer (TA-XT2i Stable Micro Systems, Godalming, Surrey, UK) was used to measure tensile strength (TS), elongation at break (E), and elastic modulus (EM) according to ASTM D882-10 (2010). Grip separation was 5 cm and cross head speed was 100 mm/min. TS was calculated from the ratio of maximum force exerted on the film during fracture to cross-sectional area, E was expressed as percentage of length increase of the EF at break, and EM was calculated from the initial slope of the stress-strain curve.

Microstructure of Films

EF samples were gold coated (EM-ACE200, Leica, Wetzlar, Germany), and scanning electron microscopy (SEM) (EVO-50, Carl Zeiss UltraPlus, Oberkochen, Germany) was used to visualize their microstructure.

Antimicrobial Assays

Minimal Fungicidal Concentration (MFC)

The individual inhibitory effect of LAE (100 to 1600 mg/L) and NAT (10 to 160 mg/L) was evaluated against *R. stolonifer*, *C. gloeosporioides*, and *B. cinerea*.

The antimicrobial assay was carried out in test tubes containing PDB, 10^6 CFU/mL of each fungus, and tested antimicrobial concentration, in a total volume of 3 mL followed by incubation at 28 ± 1 °C for 24 h. Remaining microbial population was obtained by plate count using RBA (López-Malo et al. 2005; Pitt and Hocking, 2009; Kowalczyk et al. 2015).

Minimal Bactericidal Concentration (MBC)

The MBC of LAE against *S.* Saintpaul was determined in the range 30 to 200 mg/L. Test tubes containing 10 mL of TSB, 10^6 CFU/mL of the microorganism, and tested LAE concentrations were incubated at 37 ± 1 °C for 24 h. Microbial population was enumerated on TSA plates (López-Malo et al. 2005; Becerril et al. 2013).

Viable Staining Fluorescence microscopy was carried out using LIVE/DEAD® bacterial viability kit (Molecular Probes, Eugene, OR, USA) to visualize the effect of LAE on *S.* Saintpaul cells, according to Ercolini et al. (2006). The stock solution of the two fluorochromes was prepared with SYTO-9 (20 mM) and propidium iodide (3.34 mM) in sterile deionized water. Exponential phase cultures of *S.* Saintpaul (10^6 CFU/mL) were exposed to LAE, and filtered through 25 mm diameter and 0.4 μm pore size black polycarbonate membrane (Whatman, Clifton, NJ, USA), to which 100 μL of the fluorochromes stock solution was applied. Stained cells were kept in the darkness for 15 min at room temperature, and visualized using a fluorescence microscope (Zeiss Axioskop 40, FICT Filter, Göttingen, Germany), fitted with AxioCam MRc camera and ZEN pro 2012 imaging software version 1.1.2.0. Cells counting were performed by randomly counting 10 microscopic fields using Image J version 1.49 m software (Rasband, 2007). An untreated culture of *S.* Saintpaul was used as control.

Antimicrobial Combined Effect

Once the MFCs for each microorganism were obtained for LAE and NAT, the combined antimicrobial effect was tested. The antimicrobial challenge was conducted as in Section 2.11.1, and the effective combination of each antimicrobial was estimated by the fractional inhibitory concentration (FIC). The maximum limit of each antimicrobial concentration was the MFC, and fixed single fractions (varied from 0.1, 0.2, up to 0.9) of one antimicrobial were tested in a mixture with varying concentrations of the other antimicrobial until its minimal concentration leading to full inhibition of the three fungi was detected. In this way, we built a checkerboard including these concentrations of antimicrobial combination. Care was taken to use LAE concentrations above the MBC found against *S.* Saintpaul. FIC was calculated

as the ratio of the inhibitory concentration of a compound in the antimicrobial mixture divided by the inhibitory concentration of the compound alone (López-Malo et al. 2005; Brandt et al. 2010).

Antimicrobial Activity of Films

The effectiveness of antimicrobial agents is usually reduced when incorporated into active films because of the presence of many different compounds as compared to in vitro tests. EFs were incorporated with a combination of antimicrobial agents at concentrations 1×, 5×, and 10× (Table 1). EFs preparation was described in Section 2.6, where antimicrobial combination along with Tween 80 and BW microemulsion were incorporated following the same protocol. Antimicrobial activity was evaluated by contacting films disks of 10 mm in diameter with 10^5 CFU/mL of tested fungi in PDA plates, followed by incubation at 28 ± 1 °C for 24 h. In addition, TSA plates similarly inoculated with the pathogenic bacterium were incubated at 37 ± 1 °C for 24 h. The antimicrobial effect was determined by the inhibition zone diameter (Dashipour et al. 2015).

Table 1

Combination of natural antimicrobials incorporated into modified corn starch edible films

Levels	LAE (mg/L)	NAT (mg/L)
1×	400	80
5×	2000	400
10×	4000	800

LAE lauric arginate, *NAT* natamycin, *x* times original concentration

Statistical Analysis

Physicochemical, WVP, and mechanical analyses of EFs were done in triplicate, and the means were evaluated for statistical significance ($p < 0.05$) using Tukey's test from JMP statistical software, version 5.0.1 (Cary, NC, USA).

Results and Discussion

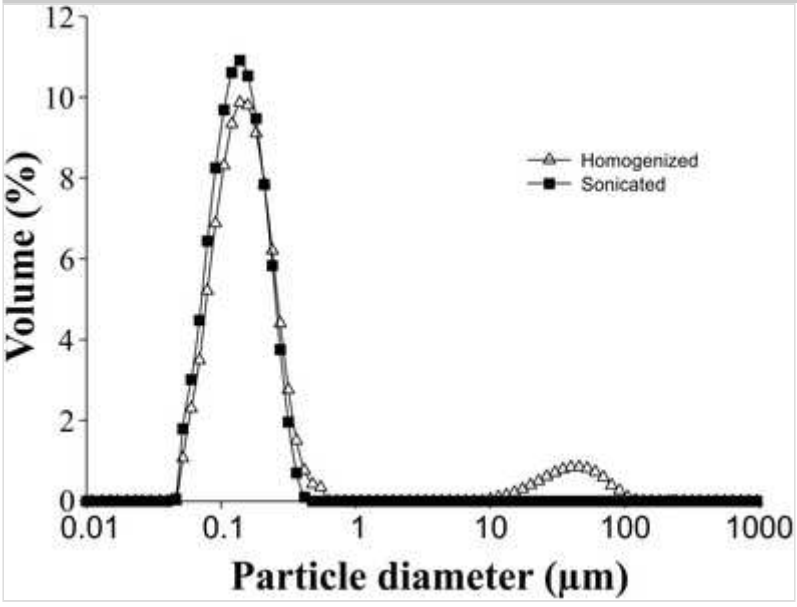
BW Microemulsion

The homogenized BW showed a particle size of 0.135 ± 0.009 μm, whereas that of the sonicated BW microemulsion was 0.117 ± 0.003 μm (Fig. 1). The homogenized

BW microemulsion showed a bimodal size distribution in which the large dispersed droplets remained heterogeneous leading to coalescence. In contrast, a particle size distribution essentially unimodal and homogeneous was obtained for the sonicated BW microemulsion, due to disaggregation of the particles as a result of the high-energy disruptive forces derived from the intense acoustic field. Particle size reduction was probably achieved as a result of the formation and collapse of micro-bubbles (Jafari et al. 2006; Kentish et al. 2008). The particle size reduction of BW microemulsion is highly dependent on selection and concentration of its components and the use of appropriate emulsifying processes (McClements and Rao 2011). The sonicated BW microemulsion did not show any phase separation after 21 days, and thus, it was selected for incorporation into the EFs formulations. The use of fatty acids (stearic, palmitic, or oleic) as emulsifiers, together with co-solvents to dissolve the complex mixture of saturated free fatty acids, mono esters, and alkanes present in BW, and the application of high energy are commonly used for microemulsion formation. Morpholine, a permitted food additive in edible coatings applied to fresh fruits and vegetables (FDA, 21 CFR 172235), has been frequently used as co-solvent (Hagenmaier and Baker 1994; Navarro-Tarazaga et al. 2008). Other co-solvents such as choloroform, *n*-decane, and acetone are not allowed in food systems, whereas some vegetable oils, ethanol, and caprilic acid are used in food systems with poor microemulsification results (Hagenmaier and Baker 1997; Flanagan and Singh 2006; Hélder et al. 2012).

Fig. 1

Particle size distribution of beeswax microemulsion using two different emulsification processes



Characterization of Starches

Amylose content of ACLS was 27.5 ± 1.5 % (*w*/*w*), whereas OS showed 15.7 ± 1.2 % (*w*/*w*). A previous work using these starches (Pérez-Gallardo et al. 2012) showed promising results on EFs thickness and WVP properties for possible application as coatings on the surface of fresh produce.

Starch modifications have allowed increased pasting clarity with lower viscosity values; acid, thermal, and high shear stability; and delayed retrogradation during storage (Kaur et al. 2006; Sánchez-Rivera et al. 2010). The acetyl content (DS = 0.142 ± 0.005 %, *w*/*w*) and the improved strength due to intermolecular binding of cross-linked commercial ACLS (94.8 ± 0.1 %, degree of cross-linking, *w*/*w*) led to homogeneous dispersions that are able to produce EFs with reduced WVP values and enhanced mechanical properties (Table 2 and Table 3). These characteristics are associated to steric hindrance caused by the attached functional groups that do not allow water to weep out, preventing re-association, in agreement with reports by Yu and Wang (2007), Hoover et al. (2010), and Gutiérrez et al. (2015).

Table 2

Physicochemical properties and water vapor permeability (WVP) of modified corn starch edibl films

Films	Beeswax (% *w*/*v*)	Thickness (μm)	Opacity (AU × nm)	Solubility (% SM)	WVP (g mm/(m^2 h kPa))
ACLS	–	50.27 ± 0.20^{e}	20.81 ± 0.40^{e}	99.57 ± 0.20^{a}	0.90 ± 0.06^{a}
ACLS	0.2	52.67 ± 0.50^{d}	23.48 ± 0.90^{cde}	97.38 ± 0.15^{c}	0.77 ± 0.01^{bc}
ACLS	0.6	55.20 ± 0.72^{bc}	27.98 ± 0.84^{c}	96.65 ± 0.07^{de}	0.69 ± 0.06^{cd}
ACLS	1.0	56.87 ± 0.70^{b}	36.75 ± 2.27^{ab}	96.03 ± 0.12^{f}	0.57 ± 0.04^{e}
OS	–	51.80 ± 0.72^{de}	22.83 ± 0.57^{de}	98.74 ± 0.22^{b}	0.82 ± 0.03^{ab}
OS	0.2	54.13 ± 0.31^{d}	26.56 ± 1.29^{cd}	97.01 ± 0.13^{cd}	0.74 ± 0.02^{bcd}
OS	0.6	57.33 ± 1.21^{b}	35.00 ± 3.02^{b}	96.82 ± 0.07^{de}	0.63 ± 0.04^{de}
OS	1.0	64.47 ± 0.95^{a}	41.15 ± 2.35^{a}	96.51 ± 0.20^{e}	0.56 ± 0.05^{e}

Mean values for the same column with different letters are significantly different ($p \leq 0.05$)

ACLS acetylated cross-linked starch, *OS* oxidized starch, *AU* absorbance units, *SM* soluble matter

Table 3

Mechanical properties of modified corn starch edible films

Films	Beeswax (% *w*/*v*)	TS (MPa)	E (%)	EM (MPa)
ACLS	–	88.53 ± 6.76^{a}	2.14 ± 1.12^{a}	320.83 ± 6.02^{a}
ACLS	0.2	68.41 ± 9.12^{bc}	3.23 ± 1.83^{a}	299.21 ± 10.08^{b}
ACLS	0.6	61.05 ± 2.73^{bcd}	4.24 ± 1.14^{a}	259.77 ± 9.63^{cd}
ACLS	1.0	51.48 ± 5.92^{cde}	4.59 ± 1.11^{a}	211.30 ± 7.85^{e}
OS	–	76.79 ± 4.84^{ab}	2.51 ± 1.17^{a}	272.86 ± 7.38^{c}
OS	0.2	62.39 ± 9.59^{bcd}	2.99 ± 1.78^{a}	246.00 ± 5.00^{d}
OS	0.6	46.28 ± 3.41^{de}	4.20 ± 1.09^{a}	214.86 ± 6.66^{e}
OS	1.0	40.96 ± 4.98^{e}	4.76 ± 1.51^{a}	203.50 ± 5.35^{e}

Mean values in the same column with different letters are significantly different ($p \leq 0.05$)

ACLS acetylated cross-linked starch, *OS* oxidized starch, *TS* tensile strength, *E* elongation at break, *EM* elastic modulus

Pastes from OS have shown low viscosity, thermal stability, and resistance to retrogradation (Kuakpetoon and Wang 2006; Sangseethong et al. 2010). The carbonyl (0.073 ± 0.005 %, *w*/*w*) and carboxyl (0.077 ± 0.006 %, *w*/*w*) content of commercial OS indicates enhanced hydrogen bonds among adjacent amylopectin molecules in a more efficient association process. These properties may be advantageously used in the preparation of dispersions leading to EFs with improved water barrier properties (Table 2), in agreement with Shah et al. (2016), and are uniform and more flexible (Table 3), as reported by Fonseca et al. (2015).

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Physicochemical Properties of Corn Starch Edible Films

Film Thickness

Thickness of EFs was 50.27 ± 0.20 μm for ACLS and 51.80 ± 0.72 μm for OS without BW, and increased up to 56.87 ± 0.70 and 64.47 ± 0.95 μm with 1 % (*w*/*w*) BW addition, for each modified starch, respectively (Table 2, column 3). Higher concentration of OS (6 %, *w*/*w*) than that of ACLS (3 %, *w*/*w*) was used to obtain suitable EFs, possibly associated to partial depolymerization during the oxidation process. This effect resulted in low viscosity, which combined with association of

carbonyl and carboxyl groups did not allow film formation upon drying at lower solids concentration. Despite the type of modified starch, incorporation of BW and its concentration significantly influenced ($p < 0.05$) film thickness, a property mainly dependent on the quantity of solids deposited per unit area (Chen et al. 2009; Muscat et al. 2013). According to Longares et al. (2004), this thickness range is unnoticed by the consumers. When using 0.2 % BW, thicknesses were reduced to 52.67 ± 0.50 μm for ACLS and 54.13 ± 0.31 μm for OS-based EFs. These values are higher than those reported by Pérez-Gallardo et al. (2012) working on similarly modified starches, giving 36.3 μm for ACLS and 40.1 μm for OS-based EFs. However, this report used poorly incorporated BW, resulting in heterogeneous EFs surface.

Film Opacity

Transparent films are characterized by lower opacity values, which represent a critical property if films are intended to be used as food coating.

The lowest opacity was obtained with 0.2 % BW (23.48 ± 0.90 AU × nm for ACLS and 26.56 ± 1.29 AU × nm for OS) (Table 2, column 4), and similar results were reported by Pérez-Gallardo et al. (2012) showing 28.8 AU × nm and 23.2 AU × nm, for ACLS and OS with 0.2 % BW films, respectively. In contrast, high opacity was shown by the 1 % (*w*/*w*) BW. The opacity is higher using OS than ACLS because of the higher concentration of OS in the EFs, which increased with BW concentration. These results suggest that opacity is strongly related with BW concentration, where the presence of non-miscible lipid micelles promotes opacity as a function of the differences in the refractive index of the phases and particle size of the dispersed phase (Fabra et al. 2009; Monedero et al. 2009). Opacity values were lower than those reported for tapioca starch/decolorized hsian-tsao leaf gum films containing emulsified BW (10 % *w*/*w*) showing much higher opacity (148 AU × nm) (Chen et al. 2009). On the other hand, acetylated corn starch-glycerol films prepared without any hydrophobic agent showed opacity values up to 115.12 AU × nm (López et al. 2008).

Film Solubility

All EFs were highly soluble (96.03 to 99.57 %), whereas those containing 1 % *w*/*w* of BW showed the lowest solubility (96.03 ± 0.12 % for ACLS and 96.51 ± 0.20 % for OS) (Table 2, column 5). Integrity loss of EFs could be due to the chemical composition and structural characteristics of starch modification, for instance ACLS may have facilitated water percolation within amorphous regions allowing

loss of soluble material. Structural weakening of OS granules, and partial depolymerization after oxidation, probably led to its increased water solubility (López et al. 2010; Xiao et al. 2012). It is known that plasticizers interact with EFs matrix, leading to increased space between chains, facilitating water migration into the EFs. This effect has been reported by Tongdeesoontorn et al. (2011), in which cassava starch films plasticized with glycerol showed high solubility (73 %). López et al. (2008) found for EFs made with acetylated corn starch (5 % *w*/*v*) plasticized with 2.5 % glycerol a solubility of 18.96 % (*w*/*v*), which was lower than that obtained here, associated to lower amylose content (11.67 % *w*/*w*). On the other hand, Kowalczyk et al. (2015) found a moderate solubility of 52.17 % (*w*/*w*) for oxidized potato starch EFs, indicating that heating was required for complete dissolution.

Water solubility of EFs may be useful for coated fresh produce or ready-to-eat foods since they will readily dissolve when tasted by the consumer remaining undetected (Nouri and Nafchi, 2014). Zamudio-Flores et al. (2009), working with EFS made from oxidized and acetylated banana starch, found that film solubility decreased with storage time (up to 120 days) due to amylopectin reorganization increasing starch crystallinity, leading to lower solubility.

Water Vapor Permeability (WVP)

When BW concentration increased, WVP significantly decreased, and EFs incorporated with the highest BW concentration (1 % *w*/*w*) showed the lowest WVP of 0.57 ± 0.04 g mm m^{-2} h^{-1} kPa^{-1} for ACLS, and 0.56 ± 0.05 g mm m^{-2} h^{-1} kPa^{-1} for OS (Table 2, column 6). ACLS produced more permeable films than OS, which may be explained by more open film structure (Pérez-Gallardo et al. 2012). Muscat et al. (2013) reported similar results for high amylose corn starch films using Tween 80 as surfactant, obtaining WVPs of 0.27 g mm m^{-2} h^{-1} kPa^{-1}, and 0.51 g mm m^{-2} h^{-1} kPa^{-1} for 5 % (*w*/*w*) and 10 % (*w*/*w*) BW, respectively. Opposite results were reported by Han et al. (2006), where a slight decrease in WVP was observed for pea starch EFs added with 10 to 40 % (*w*/*w*) BW, obtaining WVPs of 7.49 g mm m^{-2} h^{-1} kPa^{-1} to 6.59 g mm m^{-2} h^{-1} kPa^{-1}, and presence of scattered BW aggregates on the films. The higher WVP values obtained by these authors may be due to the many spaces and channels showed by the films, enough to allow penetration of water vapor through the starch matrix. In addition, the little decrease in WVP after BW incorporation suggests that the homogenization process should be carefully controlled to obtain homogeneous films showing enhanced water vapor resistance. Navarro-Tarazaga et al. (2008) produced a bilayer

consisting of BW emulsified with fatty acids on top a hydrophilic film based on hydroxymethyl propyl cellulose (HPMC), obtaining higher WVP than using a single HPMC film incorporated with the emulsified hydrophobic agents. They concluded that final lipid distribution in the film was related to their ability to form stable emulsions. These reports suggest that in the design of EFs, BW incorporated as microemulsion leads to homogeneous films with increased moisture transfer barrier, suitable for use as fresh produce coatings.

Mechanical Properties

EFs based on ACLS or OS containing increasing concentrations of BW (0 to 1 % *w*/*w*) showed enlarged E (2.14 ± 1.12 to 4.76 ± 1.51 %), whereas the opposite effect was observed for TS (88.53 ± 6.76 to 40.96 ± 4.98 MPa) and EM (320.83 ± 6.02 to 203.50 ± 5.35 MPa) (Table 3). These results show that mechanical properties of starch EFs are dependent on additives-matrix interactions, where upon increasing BW concentrations its hydrocarbons and esters interacted with the starch-sorbitol network, by acting as plasticizer (Sohail et al. 2006; Fabra et al. 2008). Thus, increased BW allowed EFs exhibiting good flexibility, low brittleness, and improved stretching capacity, resulting in better mechanical properties. Santos et al. (2014) reported that increased incorporation of carnauba wax/surfactant ratios in cassava starch EFs promoted decreased TS (23.21 to 2.89 MPa) and EM (15.16 to 5.30 MPa), while increased E (5.53 to 41.29 %). This behavior was also observed by Muscat et al. (2013) for high amylose starch-glycerol films, when BW was incorporated at 10 % *w*/*w* emulsified with Tween 80. In contrast, these authors were unable to report the mechanical properties of films when BW was incorporated at lower concentration (5 % *w*/*w*), due to phase separation during film drying. Opposite effect was observed by Chiumarelli and Hubinger (2012) working with cassava starch EFs added with carnauba wax, where TS and E decreased, while EM increased. This effect could be due to the discontinuity in the films' polymer matrix caused by carnauba wax/stearic acid ratio of 0.20:0.80 % *w*/*w* used, and a deficient emulsification process leading to flocculation, which resulted in inflexible and brittle films. Despite these authors' use of carnauba wax, concentrations were lower than those used in this work.

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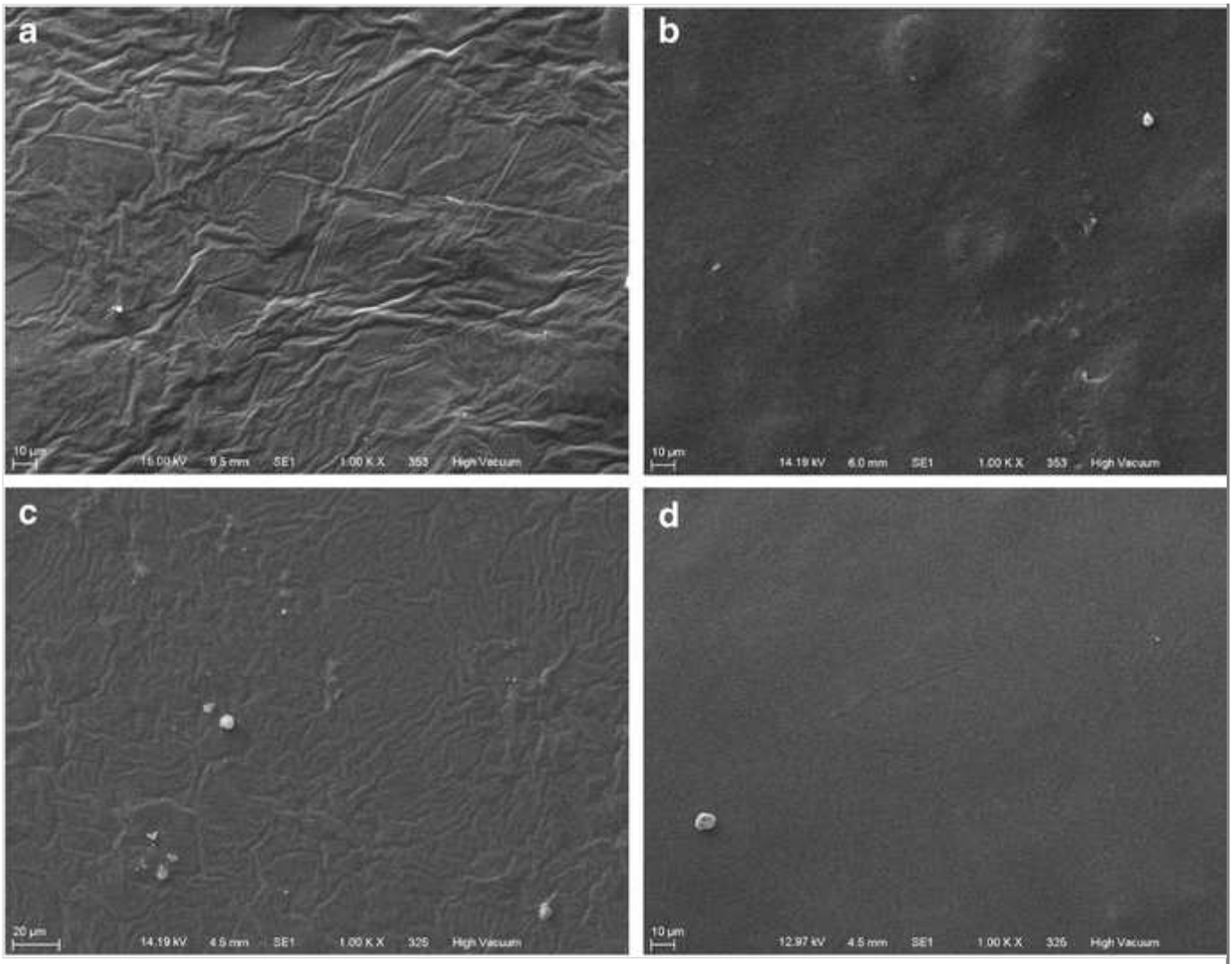
Microstructure

Corn starches (ACLS or OS) were able to support the micro-emulsified BW and formed continuous EFs without separation of the different components, pores or cracks, easy to handle, indicating an adequate homogenization. Control ACLS film

surface exhibited high roughness and disorganized structure, whereas control OS film showed a compact structure (Fig. 2a, b). EFs added with BW (1 % *w*/*w*) produced continuous and homogenous surfaces, revealing good BW incorporation into the starch polymeric matrix (Fig. 2c, d), as a result of the high-energy emulsification process applied to the microemulsion, and could be related to the improvement of water barrier and mechanical properties of EFs. Pérez-Gallardo et al. (2012) working with EFs based on ACLS, and OS added with 0.2 % (*w*/*w*) of BW emulsion, showed partial separation of BW particles on the surfaces, due to instability and heterogeneous incorporation of BW into starch EFs. In addition, Muscat et al. (2013) reported the effect of direct addition of BW (5–10 % *w*/*w*) without emulsification on surface morphology of high amylose starch EFs, resulting in evident roughness due to aggregation of wax droplets. From physicochemical, barrier, and mechanical properties of ACLS and OS films (Table 2 and Table 3), the EFs incorporated with 1 % BW were chosen to evaluate the addition of antimicrobials. These EFs gave the lowest values of WVP, TS, and EM, whereas showing increased E, without affecting thickness or opacity, producing a homogeneous surface as compared to EFs without BW.

Fig. 2

Micrographs of corn starch edible films. **a** ACLS-based films. **b** OS-based film. **c** ACLS-based film added with 1 % (*w*/*w*) beeswax (BW). **d** OS-based film added with 1 % (*w*/*w*) BW



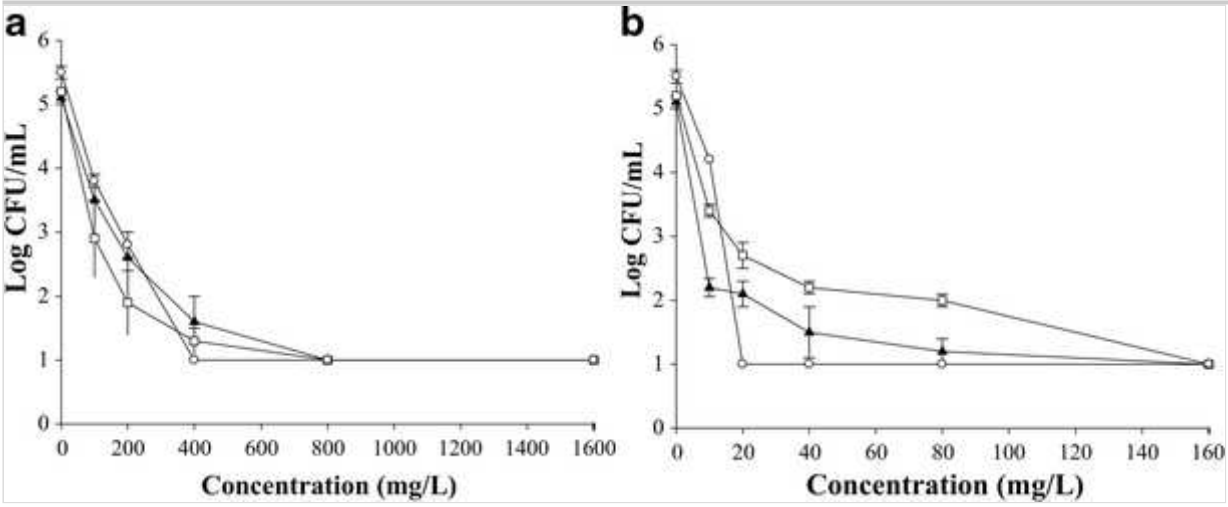
Minimal Fungicidal Concentration (MFC) and Minimal Bactericidal Concentration (MBC)

MFC of LAE on *R. stolonifer*, *C. gloeosporioides*, and *B. cinerea* was 800 mg/L (Fig. 3a), and MFC of NAT was 160 mg/L (Fig. 3b), with population reduction of 4.3 log CFU/mL (method limit equal to 1 log) in both cases. No reports were found about the use of LAE and NAT against these fungi. However, Higueras et al. (2013) found MFC of LAE against *Aspergillus niger* (320 mg/L), *Penicillium chrysogenum* (280 mg/L), and *Cladosporium cladosporioides* (80 mg/L). On the other hand, Delves-Broughton et al. (2005) reported a much smaller MFC of NAT against *B. cinerea* (25 mg/L), which could be due to the specific strain used in this work (CDBB-H-1556), and the low fungal inoculum (10^4 CFU/mL) used by those authors.

Fig. 3

Minimal fungicidal concentration (MFC) of **a** arginate lauric (LAE) and **b** natamycin (NAT). *R. stolonifer* (*black triangle*); *C. gloeosporioides* (*white square*); *B. cinerea*

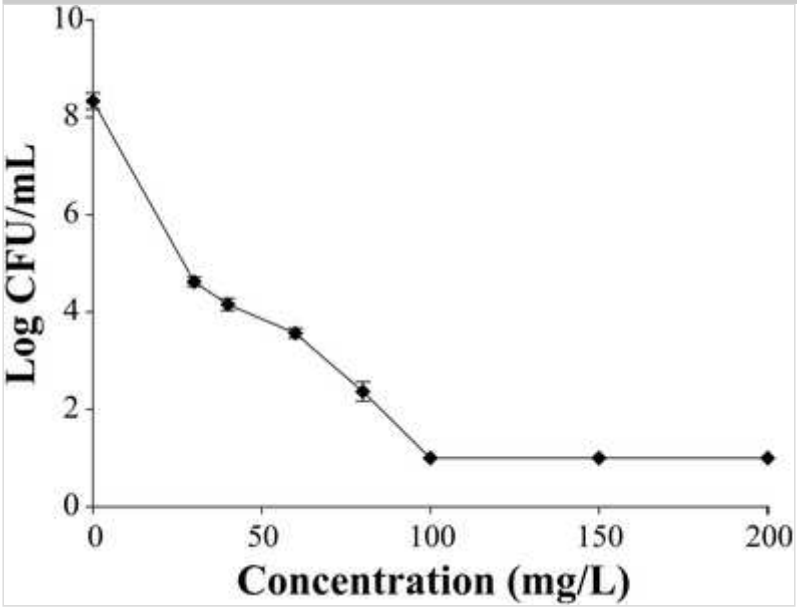
(*white circle*)



The use of LAE at 100 mg/L achieved complete inhibition (MBC) of *S.* Saintpaul, reducing 7.3 log CFU/mL (Fig. 4). Lower concentrations for *Salmonella enterica* inhibition were found by Becerril et al. (2013) and Ma et al. (2013), showing MBC of 25 mg/L and 23.5 mg/L of LAE, respectively. These values indicate than *S.* Saintpaul is more resistant to LAE than other strains.

Fig. 4

Minimal bactericidal concentration (MBC) of lauric arginate (LAE) versus *S.* Saintpaul (*black diamond*)



Despite individual concentrations that completely inhibited the growth of the three fungi and bacterium were found in this work, they were considerably high. It is

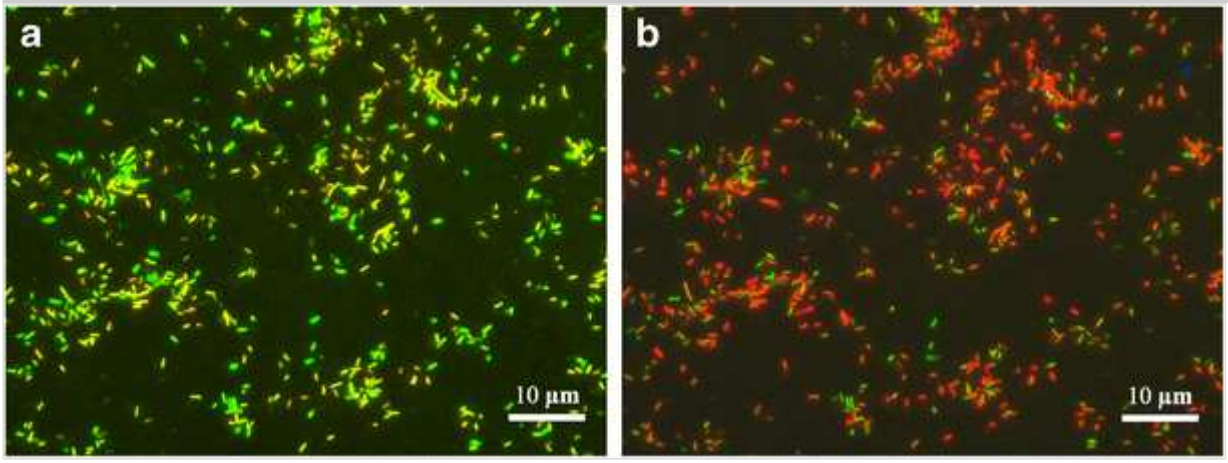
known that LAE is mainly effective against bacteria and fungi, while NAT controls fungal growth only. Thus, the hurdles concept was applied by using fractional inhibitory concentrations of these two antimicrobials trying to reduce individual concentrations, while achieving complete microbial inhibition.

Viable Staining

Using the MBC of LAE (100 mg/L) against *S.* Saintpaul, a viable staining procedure was carried using two fluorochromes, SYTO-9 (green fluorescence), which penetrates all types of cellular membranes and propidium iodide (red fluorescence), that only penetrates the bacterial membrane when it is damaged. Most untreated bacterial cells (96.4 %) were green stained and considered as live, whereas only a small proportion were considered dead, and stained red (3.6 %) (Fig. 5a). In contrast, bacteria after 15 min contact time with LAE stained red (98.8 ± 0.7 %) (Fig. 5b). Similar results are reported by Rodríguez et al. (2004) for *Salmonella typhimurium* contacted with 32 mg/L of LAE, but with a contact time of 30 min, where 90.7 % cells stained red. This fluorescence technique has been used to visualize the antimicrobial effectiveness of bacteriocin-containing polyethylene films contacted for 2 h with *Listeria monocytogenes* suspensions, showing decreased live cells (Ercolini et al. 2006). Fluorescence micrographs of *Escherichia coli* treated with 10-fold diluted basil oil nanoemulsion for 30 min showed destruction of all bacterial cells (Ghosh et al. 2013).

Fig. 5

Viable staining of *S.* Saintpaul. **a** Control cells. **b** Cells treated with LAE (100 mg/L)



Antimicrobial Combined Effect

There were many antimicrobial combinations leading to complete inhibition of the three fungi. The combination showing effective inhibition using the least antimicrobial concentration was 400 mg/L LAE (1/2 MFC) and 80 mg/L NAT (1/2 MFC) (Section 3.7), without affecting the MBC for *S.* Saintpaul. The FIC_{index} was calculated as FIC (LAE) + FIC (NAT) = 0.5 + 0.5 = 1, indicating an additive effect, which occurs when two combined antimicrobials give inhibition equivalent to the sum of each antimicrobial acting independently. FIC value <1 indicates a synergistic effect, while FIC >1 indicates an antagonistic effect (López-Malo et al. 2005). Ma et al. (2016) reported a synergistic antimicrobial effect of the combination of LAE and cinnamon oil on *L. monocytogenes*, but antagonistic effect on *E. coli* and *S. enterica*. EDTA incorporation enhanced the activity of LAE and overcame the antagonistic effect of LAE-cinnamon oil combination. In addition, Brandt et al. (2010), working with *L. monocytogenes*, reported an additive inhibition effect of a combination of nisin and LAE, a synergistic effect of nisin and acidic calcium sulfate, while ε-poly-L-lysine combined with acidic calcium sulfate produced an antagonistic effect.

Antimicrobial Effect of ACLS- and OS-Based EFs

ACLS or OS EFs added with 1 % *w*/*w* BW that showed the best compromise among physicochemical, barrier, and mechanical properties (Table 2 and Table 3) were selected to incorporate the effective antimicrobial combination (400 mg/L LAE, and 80 mg/L NAT) at different levels (Table 1). Inhibitory zones of antimicrobial EFs were only observed when the effective concentration of antimicrobial combination was increased 5 times or higher. The increased antimicrobial concentration required to exert their effect once incorporated into EFs has been reported previously (Higueras et al. 2013), and could be due to higher diffusivity in liquid form, as compared to their diffusion within the EFs, where interaction with its components hinders their effect. Inhibition zones of ACLS EFs were 20.0 ± 2.0 mm for *R. stolonifer*, 29.7 ± 1.9 mm for *C. gloeosporioides*, 22.7 ± 2.0 mm for *B. cinerea*, and 14.0 ± 1.5 mm for *S.* Saintpaul (Fig. 6). The same effect was observed by OS EFs 25.7 ± 1.0 mm for *R. stolonifer*, 27.3 ± 2.4 mm for *C. gloeosporioides*, 20.5 ± 1.2 mm for *B. cinerea*, and 14.0 ± 1.5 mm for *S.* Saintpaul (Fig. 7). Much higher concentrations of LAE (10 to 100 g/L) were added to chitosan films to achieve inhibition of *S. enterica* (11.9 ± 0.9 to 14.2 ± 0.4 mm) (Higueras et al. 2013). Despite using these high LAE concentrations, the latter authors could not inhibit any of the following fungi: *A. niger*, *C. cladosporioides*, and *P. chrysogenum*, because LAE migration was probably not enough to produce inhibition. Cong et al. (2007) showed

antimicrobial activity of a bilayer film based on chitosan and polyethylene-wax microemulsion, each containing 20 mg/L NAT against *Alternaria alternate* and *Fusarium semitectum*, which cause Hami melon decay during storage. Another study using EFs based on chitosan and alginate added with 600 mg/L NAT inhibited cheese deteriorative fungi (*Debaromyces hansenii*, *Penicillium commune*, and *Penicillium roqueforti*) (Da-Silva et al. 2013).

Fig. 6

Antimicrobial effect of ACLS edible films against **a** *R. stolonifer*, **b** *C. gloeosporioides*, **c** *B. cinerea*, and **d** *S.* Saintpaul

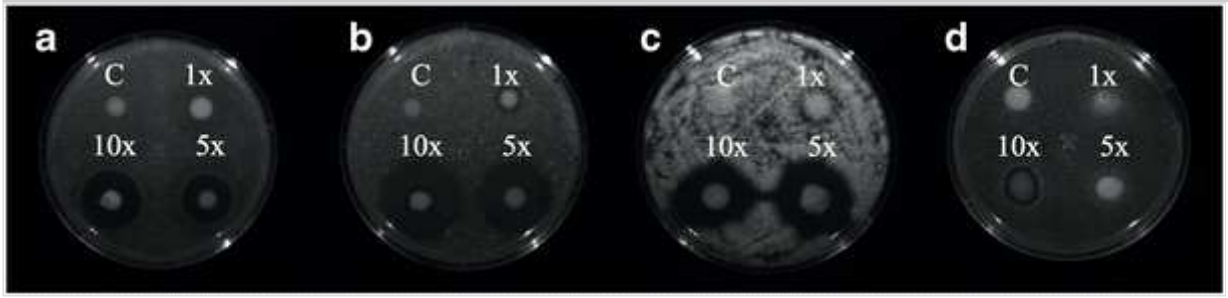
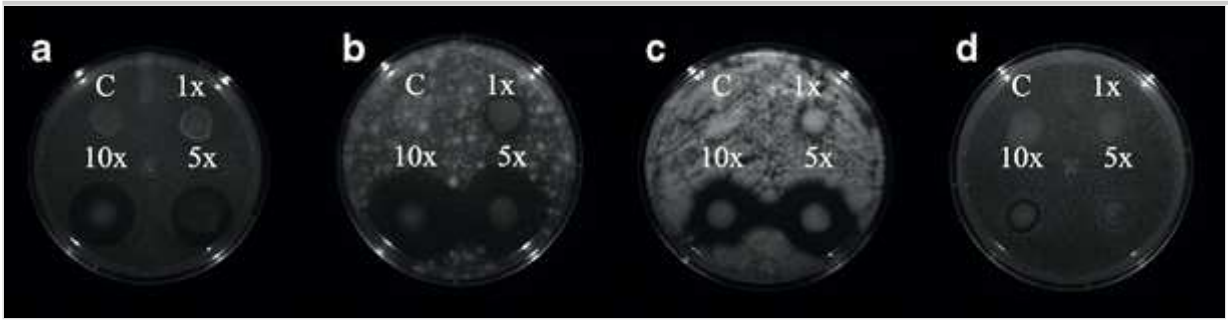


Fig. 7

Antimicrobial effect of OS edible films against **a** *R. stolonifer*, **b** *C. gloeosporioides*, **c** *B. cinerea*, and **d** *S.* Saintpaul



Final concentration of antimicrobial EFs was 1.0 μg/cm^2 of LAE and 0.2 μg/cm^2 of NAT. These are the required amounts to prevent the growth of the three fungi and the pathogenic bacterium studied, and thus keeping food produce safe. Films are thin, and contribute sparingly to the total weight of the food material, and therefore antimicrobial concentrations will not exceed the maximum permitted limits of 225 mg/kg for LAE (EFSA, 2007) and 25 mg/kg for NAT (Delves-Broughton et al. 2005).

Conclusions

Filmogenic dispersions using ACLS and OS as major components are able to form EFs with good visual, physicochemical, and mechanical properties. They represent an excellent alternative for further use as coatings to increase shelf life of fresh produce, without perception by the consumers. BW was incorporated into the modified starch films as a microemulsion (1 % *w*/*w*), offering homogeneous EFs surface, lower WVP, while improved mechanical properties, without affecting thickness or opacity, which may have an impact on the preference of coated foods. An additive effect resulted from the combined use of the natural antimicrobial LAE and NAT, leading to a substantially decreased concentration requirement of each substance without affecting their individual effectiveness. However, increased antimicrobial concentration (2000 mg/L of LAE plus 400 mg/L of NAT) was needed when incorporated into EFs, probably due to low diffusivity within the solid coating. Antimicrobial EFs led to complete inhibition of the fresh foods deteriorative fungi *R. stolonifer*, *C. gloeosporioides*, *B. cinerea*, and of *S.* Saintapul, which is a common pathogenic bacterium of fresh produce.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interests.

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