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Elaboration and characterization of pectin-gellan films added with concentrated supernatant of *Streptococcus infantarius* fermentations, and EDTA: effects on the growth of *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* in a Mexican cheese medium, and physical-mechanical properties

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Abstract

Films were prepared with 0.2% (w/v) gellan gum, 1% (w/v) citric pectin, 0.5% (w/v) glycerol, 5 mM $CaCl_2$, 0.05 M Ethylenediaminetetraacetic acid (EDTA), and different concentrations of an antimicrobial activity-concentrated supernatant (AMC) obtained from fermentations of *Streptococcus infantarius*, which produces bacteriocin-like inhibitory substances (i.e., treatments F1, F2 and F3 with 75, 90 and 120 arbitrary units (AU)/mL, respectively). The treatments were based on a minimum-inhibitory-concentration (i.e., AMC, 90 AU/mL, plus EDTA, 0.05 M) for *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* growing in brain-heart-infusion medium at 35 °C. The films hindered the bacterium growth in selective media: *E. coli*-MacConkey, *S. aureus*-Baird Parker and *L. monocytogenes*-Oxford, during 30 days at 25 °C. Moreover, the F2 films entirely inhibited the growth of the tested bacteria in a Mexican cheese medium, in 7-day cultures at 35 °C; contrariwise, controls with no film exhibited bacterial growths in the range 10^7-10^9 CFU/g. The physical-mechanical properties of the films were changed by the addition of EDTA-AMC, being (F2 film)/(control-film with no AMC nor EDTA): Young's modulus (MPa), 1,394/707; Elongation at break (%), 1.9/9.3; Stress at break (MPa), 5.7/12.6; Water Vapour Permeability (10^{-11} g m Pa^{-1} s^{-1} m^{-2}), 3/20, and Oxygen Permeability (10^{-12} g m Pa^{-1} s^{-1} m^{-2}), 1.9/1.2.

Keywords: lactic acid bacterium; bacteriocins; biopreservation; mechanical properties; gas permeability.

Practical Application: Development of a biodegradable-film containing pectin, gellan, EDTA and antimicrobial substances produced by *Streptococcus infantarius*, for an effective inhibition of pathogen bacteria like *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*, that would contaminate the surface of foods like Mexican fresh cheese.

1 Introduction

The food packaging research is of worldwide interest due to food safety matters, among others (Campos et al., 2010). Specifically, research dealing with the packaging of dairy products, particularly cheese, encompasses studies concerning both fresh and ripened cheese; for example, Brazilian "Prato" cheese (Marques et al., 2017); Spanish "Zamorano" ripened sheep cheese (Otero et al., 2014), and Egyptian soft white cheese (Youssef et al., 2016), among others. Moreover, foodborne disease episodes due to cheese consumption have occurred affecting people. Examples of this are: cheese related listeriosis outbreaks in Austria, Germany and Czech Republic (Fretz et al., 2010), Portugal (Magalhães et al., 2015) and USA (Silk et al., 2013); *Escherichia coli* O157:H7 infection associated with fresh cheese curds in Wisconsin (Durch et al., 2000); *Staphylococcus aureus* food poisoning outbreaks linked to raw milk semi-hard, raw milk soft, sheep's milk and sliced soft cheese in France (13 outbreaks were reported by Kérouanton et al. (2007) between 1981 and 2002).

Concerning the antimicrobial packaging for the bioconservation of cheese, one alternative is the use of biodegradable polymer films added with bacteriocins as natural antimicrobial agents (Marques et al., 2017; Ollé Resa et al., 2016). Bacteriocins are small peptides, ribosomally synthesized by certain bacterial strains, that are active against other bacteria, with multiple biotechnological applications including food conservation, being nisin the only bacteriocin with GRAS status (López-Cuellar et al., 2016). In order to increase the antimicrobial spectrum in bacteriocin applications, they are frequently used in combination with other agents, as the chelator Ethylenediaminetetraacetic acid (EDTA), which contributes to make the bacterium outer membranes more permeable. Thus, the use of bacteriocins in combination with EDTA would be more effective against bacteria, including the inhibition of Gram negative bacteria like *Escherichia coli* (Vaara, 1992). Furthermore, some biopolymers do exhibit an antimicrobial activity, that would contribute to a more effective antimicrobial food packaging. This is the case of pectins (Calce et al., 2014; Jindal et al., 2013) and chitosan (Aider, 2010).

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The present article reports the main results concerning the preparation and characterization of films containing gellan gum and citric pectin, added with EDTA and antimicrobial activity-concentrated supernatant (AMC) from fermentations of the lactic acid bacterium, *Streptococcus infantarius*, which produces bacteriocin-like inhibitory substances. The films were tested against *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* grown in specific selective media and in a Mexican fresh cheese medium. Several film physical-mechanical properties were also determined: Young's modulus, stress and elongation at break, water vapour permeability and oxygen permeability, all of them important to consider in defining possible food packaging applications.

2 Materials and methods

2.1 Biological specimens

Streptococcus infantarius was kindly provided by C. Wacher-Rodarte (FQ-UNAM, Mexico). This strain was isolated from *Pozol*, a traditional fermented Mexican beverage (Tavera-Montes, 2010).

Escherichia coli ATCC-25922, *Staphylococcus aureus* ATCC-25923 and *Listeria monocytogenes* CFQ-103, were kindly provided by G. Díaz-Ruiz (FQ-UNAM, Mexico).

All bacteria were conserved at -80 °C in 20% v/v glycerol.

2.2 Culture media

Brain heart infusion broth, BHI (Bioxon® México). MacConkey Agar (Sigma-Aldrich). Baird Parker Agar (BD™ DIFCO, France). Oxford Agar (BD™ DIFCO, France). De Man-Rogosa-Sharpe broth, MRS (BD™ DIFCO, France).

Mexican fresh cheese medium (MCh): 4.4% (w/v) Mexican fresh cheese (Dairy Products Workshop, ICAp-UAEH), 1.5% (w/v) bacto-agar (BD™ DIFCO, France). The cheese used was made with pasteurised cow milk, yielding an average of 0.84% (w/v) protein and 0.1% (w/v) fat (Hnosko et al., 2009) into the whole medium.

2.3 Functional additives and reagents

A batch of an antimicrobial activity-concentrated supernatant (AMC) from a three litter-*S. infantarius* fermentation, was obtained according to López-Ortega (2014). The AMC contained bacteriocin-like inhibitory substances with an antimicrobial activity of 6,400 arbitrary units (AU)/mL, determined by the critical dilution method (Nuñez et al., 1996; Sánchez-Reyes, 2014).

Ethylenediaminetetraacetic acid (EDTA) disodium salt dehydrate, Sigma-Aldrich, Lot# SLBM9213V. Citric pectin, GENU® Pectin, DE=36%, CP-Kelco, USA. Low acyl Gellan gum, Kelcogel, CP-Kelco, USA. Glycerol, Química Meyer, Mexico. Calcium chloride ($CaCl_2$) JT Baker, Mexico.

2.4 Minimal inhibitory concentration of the functional additives

The determination of the minimal inhibitory concentration of AMC with EDTA, was carried out based on procedures described by Wiegand et al. (2008). Triplicate wells of microtiter plates (Corning® Costar®) were filled to 220 µL per well, with blends of 24 h-old culture broths of bacteria (*E. coli*, *S. aureus* or *L. monocytogenes* in BHI), AMC, EDTA and fresh BHI, to give initial concentrations of 4×10^4-7×10^4 bacterial colony-forming units (CFU)/mL, in presence of the combination of AMC and EDTA at concentrations of 0, 75, 90, 200 and 400 AU/mL, and 0, 1×10^{-3}, 2×10^{-3}, 4×10^{-3}, 8×10^{-3}, 2×10^{-2} and 5×10^{-2} M, respectively. Then, plates were incubated at 35 °C and bacterial growth inhibition was evaluated after 24 h.

2.5 Preparation of the antimicrobial films

Three functional film treatments were tested (F1, F2 and F3) and the control (FC). All filmogenic solutions contained 1% (w/v) citric pectin, 0.2% (w/v) Gellan gum, 0.5% (w/v) glycerol and 5 mM $CaCl_2$. The F1, F2, F3 and FC films contained [EDTA (M)/AMC (AU/mL)] ratios of 0.05/75, 0.05/90, 0.05/120 and 0/0, respectively. The film preparation method was based on a previous report (Calderón-Aguirre et al., 2015). The polymers were dissolved in distilled water and heated at 60-65 °C during 40 min, to add glycerol and $CaCl_2$; then increasing heat to rise 75 °C. Mixing continued another 30 min; followed by the addition of the antimicrobial additives. Then, each batch of 645 g of filmogenic solution was poured into a Teflon® cast (EKCO®, 39.5×27×2 cm) and dried in an oven (Shel-Lab, 1380FX) at 35 °C during 17 h.

The prepared films were then conditioned into a desiccator during 48 h at 50-55% RH and 23 °C. The average thickness of films was measured at five points each one with a micrometer to the nearest 0.0001 mm (Truper, Mexico).

2.6 Antimicrobial film effects on Escherichia coli, Staphylococcus aureus and Listeria monocytogenes growing in selective media

Escherichia coli, *S. aureus* and *L. monocytogenes* were grown separately in cap tubes with 5 mL of BHI during 24 h at 35 °C; then, appropriate dilutions of each bacterium culture were done using sterile isotonic salt solution, 0.9% (w/v) NaCl, to spread 200 µL of a convenient dilution inoculating 25 CFU per plate (Interlux, 60×15 mm) with the corresponding selective medium: MacConkey, Baird Parker and Oxford, respectively. Then, each inoculated agar plate was covered with a 6 cm-diameter circular film, previously disinfected with UV radiation during 24 h (12 h each side) in a Labconco® Class II Type A2 safety cabinet. Later on, the cultures were incubated at 25 °C during 30 days in a Ríos-Rocha S.A. EC-33 incubator with a mean relative humidity, RH, of 40%. The films F1, F2, F3 and FC, and a control without film (NF) were tested by triplicate.

2.7 Antimicrobial film effects on Escherichia coli, Staphylococcus aureus and Listeria monocytogenes growing in a Mexican fresh cheese medium

Escherichia coli, *S. aureus* and *L. monocytogenes* were grown separately in cap tubes with 5 mL of BHI during 24 h at 35 °C; then proper dilutions of each bacterium culture were done using sterile isotonic salt solution for spreading 200 µL of an appropriate dilution having 100 CFU, per plate (Interlux, 60×15 mm) containing MCh medium. Later, the surface of

the inoculated plates was covered with 6 cm-diameter circular films, previously disinfected with UV radiation during 24 h (12 h each side) in a Labconco® Class II Type A2 safety cabinet. The cultures were then incubated at 35 °C for 7 days, taking samples at t=0, 1, 3, 5 and 7 days, by triplicate. Every sample (1 plate containing 5 mL agar) was homogenized within 45 mL peptone water in a Seward Stomacher® 400 Circulator, at 300 rpm during 5 min. Later, viable counts were prepared by homogenate-decimal dilutions, then mixing 1 mL samples into BHI soft agar plates (Interlux, 90×15 mm) to be incubated at 35 °C during 24 h. Based on results obtained in Section 2.6, both F2 and FC treatments were tested, and bacterium-inoculated (NF) plates with no film as controls; all carried out by triplicate. The initial viable count (X_0; CFU/g), the maximum bacterium concentration (X_{max}; CFU/g), the multiplication factor ((X_{max}/X_0), dimensionless) and the maximum growth rate (μ_{max}; h^{-1}) were computed for each tested bacterium.

2.8 Mechanical characterization of the antimicrobial films

The mechanical characterization of the F2 and FC films was done according to the ASTM D882-10 Standard Test Method (American Society for Testing and Materials, 2010) in a Texture Analyser TA plus Lloyd. Every film sample was cut into the "dog-bone" shape (Type M-I tension test specimen) according to the standard ASTM D638M-93 (American Society for Testing and Materials, 1993). The samples were then conditioned during 48 h at 50-55% RH and 25 °C. Afterwards, samples were gripped in the texture analyser with an initial separation of 0.05 m. The tensile tests were carried out at a cross head speed of 1 mm/s. At least 30 replicates per film treatment were carried out, to determine the stress-Hencky strain function of the samples through the force-distance data. Specimens that failed at the grip contact point were discarded. The Young's modulus (EM; MPa) was determined through the slope of the linear region of the stress-strain curves. The ultimate mechanical properties of the films, stress ($\sigma_{T,max}$; MPa) and elongation at break (E_{max}; %) were determined at the rupture point (Calderón-Aguirre et al., 2015).

2.9 Water vapour permeability of the antimicrobial films

The water vapour permeability (WVP) of F2 and FC films was determined according to the standard method ASTM E96-00 (American Society for Testing and Materials, 2000). Film disks, previously equilibrated at 53% RH and 25 °C during 48 h, were mounted on aluminium permeation cells containing silica gel (0% RH); then the cups were placed within a cabinet which was previously equilibrated at 75 ± 2% RH and 23 ± 2 °C during 24 h. Later, the cups were weighed every hour during 8 h. Four runs were done per treatment, and the WVP values were determined according to Aguirre-Loredo et al. (2014).

2.10 Oxygen permeability of the antimicrobial films

The oxygen permeability (PO_2) of the films F2 and FC was determined in accordance to the standard ASTM D1434-82 (American Society for Testing and Materials, 1982) by means of a film-package permeability tester (Labthink VAC-V2, China). Films were conditioned at 50-55% RH during 48 h to be mounted into the chambers of the tester. The tests were performed at 25 °C, using research-grade high-purity (99.998%) oxygen gas (34161, INFRA® México). All runs were carried out in the quadruple precision.

2.11 Statistical analysis

All data are presented as the mean ± standard deviation of each treatment. Data were analysed for statistical significance through the analysis of variance (ANOVA) followed by the Tukey test ($p<0.05$). Differences between pairs of means were assessed through the t-test ($P < 0.05$) (SigmaPlot 12.5, SPSS Inc., USA).

3 Results and discussion

3.1 Antimicrobial activity of the films

The concentrated supernatant (AMC) obtained from the *S. infantarius*-culture broth exhibited antimicrobial activities against both *Listeria monocytogenes* and *Staphylococcus aureus*, but apparently did not affect *Escherichia coli*. In order to elaborate a bioactive film, capable to hinder the growth of the three bacteria (i.e., *E. coli*, *S. aureus* and *L. monocytogenes*), the metal chelator EDTA was incorporated into the filmogenic solution, due to its effects as an outer membrane permeabilization agent that can promote the release of lipopolysaccharides from the bacterium cell membrane. Thus, in the present study, it was determined a minimal inhibitory concentration of AMC, 90 AU/mL, with EDTA, 0.05 M, which hindered the growth of *E. coli*, *S. aureus* and *L. monocytogenes* in BHI medium (Figure 1). In the past,

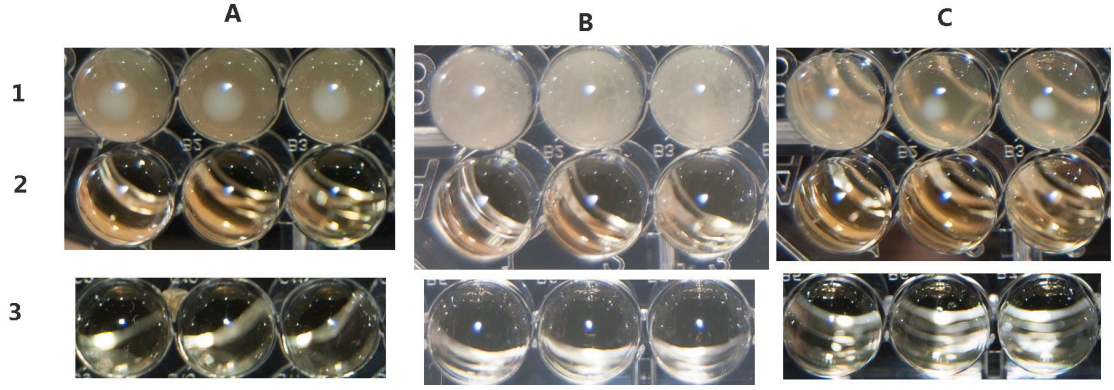

Figure 1. Minimal inhibitory concentration of AMC with EDTA for (A) *Escherichia coli*; (B) *Staphylococcus aureus*; and (C) *Listeria monocytogenes* in BHI, incubated at 35 °C for 24 h. Key: (1) AMC, 0 AU/mL; EDTA, 0 M; (2) abiotic control; (3) AMC, 90 AU/mL; EDTA, 0.05 M.

other groups have tried combinations of EDTA with conventional antimicrobials to hinder the growth of both Gram-positive and Gram-negative bacteria in various systems. For example, Sinigaglia et al. (2008) tested conditioning brines with 0.25 g/L lysozyme and 10-50 mM Na_2-EDTA in mozzarella cheese, reporting a significant inhibition of spoilage microorganisms, including coliforms. In other report, Economou et al. (2009) found that the combination of 500-1500 IU nisin with 50 mM EDTA affected the growth of Enterobacteriaceae in chicken meat. Additionally, Banin et al. (2006) found a synergic effect of Gentamicin (10 mg/mL) and EDTA 50 mM which hinder the growth of *Pseudomonas aeruginosa*.

Once an effective antimicrobial activity was obtained by combination of AMC, 90 AU/mL, with EDTA, 0.05 M, the functional films were elaborated keeping a constant EDTA concentration, 0.05 M, and testing three levels of AMC: 75, 90 and 120 AU/mL. The film matrix contained two biopolymers: low-methoxyl pectin that promotes gelation mainly due to calcium bridge ionic bonds between carboxylic groups belonging to different chains in close contact with each other (Thakur et al., 1997); and deacetylated gellan gum that develops strong gels in presence of calcium ions (Pérez-Campos et al., 2012).

Figure 2 shows the main results concerning the growth of the tested bacteria in selective media: *E. coli*-MacConkey agar; *S. aureus*-Baird Parker medium, and *L. monocytogenes*-Oxford medium, in contact with the functional films. The three bacteria grew well in control plates (NF, inoculated culture media with no films), where the counts were 78 CFU/plate and 105 CFU/plate for *E. coli* and *L. monocytogenes* after 2 days of incubation, respectively, while *S. aureus* exhibited 77 CFU/plate at the third day. These colony counts remained without significant changes till the end of the experiments. On the contrary, all film treatments (i.e., FC, F1, F2 and F3) hindered any bacterial growth during a 30 day-incubation period (Figure 2). Using an analogous approach, Mendoza-Mendoza et al. (2013) tested the antimicrobial activity of caseinate films with and without *S. infantarius*-AMC, reporting that films without AMC could not hinder the growth of *L. monocytogenes* in Oxford agar at 35 °C for 70 h; nonetheless, the antilisterial activity was effective when AMC was incorporated into the caseinate films, suggesting that caseinate alone has no antimicrobial activity against *L. monocytogenes*.

In the present work, the antimicrobial effects of the films would be attributed to a synergic interaction of AMC, EDTA and a probable contribution of gellan gum and pectin (i.e., F1, F2 and F3 treatments); nonetheless, FC films which contained gellan gum and pectin, but not AMC nor EDTA, also exhibited a notable antimicrobial activity, that would be related with the reported antimicrobial properties exhibited by several carbohydrate polymers (i.e, karaya gum, chitosan, algal polysaccharides (Ramawat & Mérillon, 2015); particularly, it has been reported the antimicrobial activity of pectins extracted from

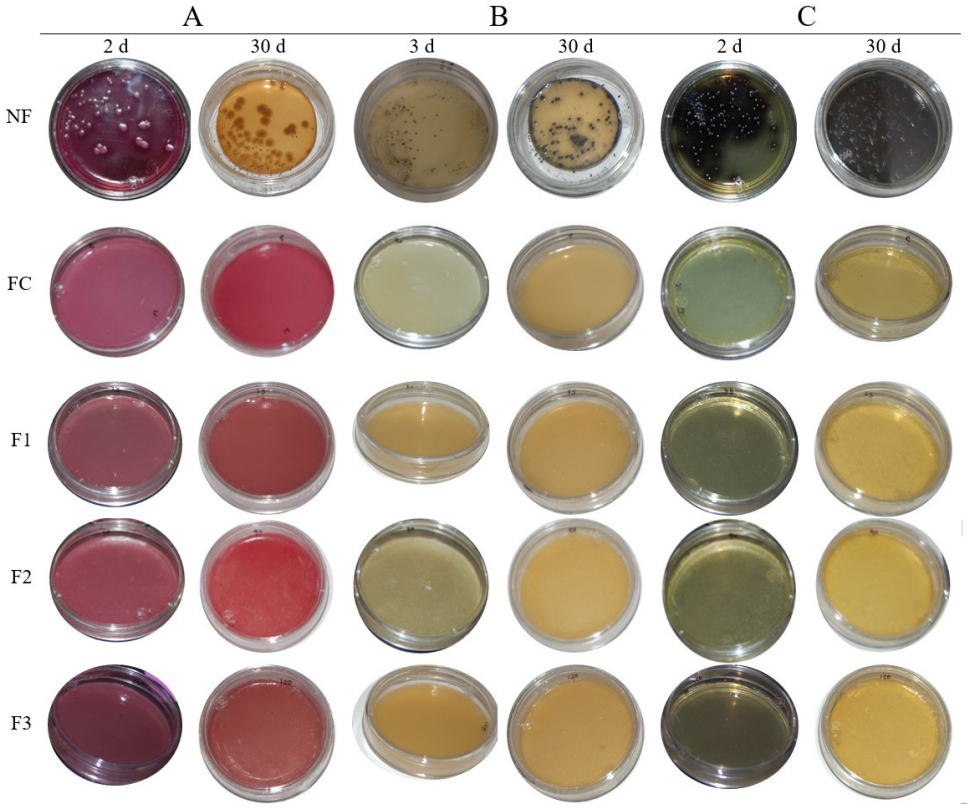

Figure 2. Growth of the tested bacteria in selective media at 25 °C and 2 or 3, and 30 days of incubation time. (A) *Escherichia coli*-MacConkey agar; (B) *Staphylococcus aureus*-Baird Parker medium; and (C) *Listeria monocytogenes*-Oxford medium. The bacteria grew well in NF plates (only culture media, no films). No bacterial growth was apparent in plates with films FC (base films containing pectin and gellan gum), as well as with films F1, F2 and F3, which were elaborated with base film-forming solution, EDTA 0.05 M, plus AMC at 75, 90 and 120 AU/mL, respectively.

apple peel (both pristine and modified samples) against *E. coli* and *S. aureus* (Calce et al., 2014), as well as that of *Aegle marmelos* fruit against *Bacillus cereus* and *E. coli* (Jindal et al., 2013). The antimicrobial activity exhibited by the FC films would be attributed mainly to its pectin content, directly associated to the uronic acid content within the polymer (Jindal et al., 2013), as well as the degree of esterification of carboxyl group of galacturonic acid in pectin molecules (Krivorotova et al., 2017). Gullón et al. (2013) also have attributed valuable antimicrobial effects to the pectic oligosaccharides.

Based on the bacterial growth inhibition obtained in the films-selective media experiments, it was decided to test only the F2 and FC treatments in MCh medium experiments. Figure 3 shows the effects of functional films on the growth of *E. coli*, *S. aureus* and *L. monocytogenes* in MCh medium.

The three tested bacteria grew well in cheese medium plates with no films (Figure 3, circle symbols). During the first day of incubation, they exhibited maximum growth rates, μ_{max}, for *E. coli*, 0.27 h^{-1} (≡6.58 day^{-1}; r^2=0.99); *S. aureus*, 0.17 h^{-1} (≡4.19 day^{-1}; r^2=0.99); and *L. monocytogenes*, 0.12 h^{-1} (≡2.84 day^{-1}; r^2=0.99). Furthermore, the bacteria achieved maximal concentrations (X_{max}) and multiplication factors (X_{max}/X_0) of 6.4×10^8 CFU/g and $X_{max}/X_0 = (6.4\times10^8)/(9.9\times10^1) = 6.5\times10^6$ times; 1.1×10^9 CFU/g and $(1.1\times10^9)/(9.9\times10^1)=1.1\times10^7$ times, and 1.7×10^7 CFU/g and $(1.7\times10^7)/(9.0\times10^1)=1.9\times10^5$ times, for *E. coli*, *S. aureus* and *L. monocytogenes*, respectively. The bacteria were cultured in a complex medium, MCh medium, containing an average of 0.84% (w/v) protein and 0.1% (w/v) fat (Hnosko et al., 2009), that supported a vigorous microbial growth involving increments of 6 to 7 log cycles in the bacterial concentrations.

On the other hand, in contrast of what occurred in selective medium cultures (Figure 2), the FC films exhibited a moderate antimicrobial activity against the three bacteria inoculated in MCh medium (Figure 3, triangle symbols). The corresponding calculated values of the average μ_{max} during the FC experiments, were 0.19 day^{-1} (r^2=0.86), 0.17 day^{-1} (r^2=0.73) and 0.26 day^{-1} (r^2=0.83) for *E. coli*, *S. aureus* and *L. monocytogenes*, respectively, which suggested moderate unfavourable conditions for the growth of the bacteria in these systems. In fact, within FC-MCh systems the tested bacteria achieved final concentrations and multiplication factors of 2.1×10^2 CFU/g and $(2.1\times10^2)/(1.0\times10^1)=2.1\times10^1$ times; 2.3×10^2 CFU/g and $(2.3\times10^2)/(2.2\times10^1)=1.0\times10^1$ times, and 5.1×10^2 CFU/g $(5.1\times10^2)/(1.5\times10^1)=3.5\times10^1$ times, for *E. coli*, *S. aureus* and *L. monocytogenes*, respectively, involving increments in the bacterial concentrations of merely 1 log cycle.

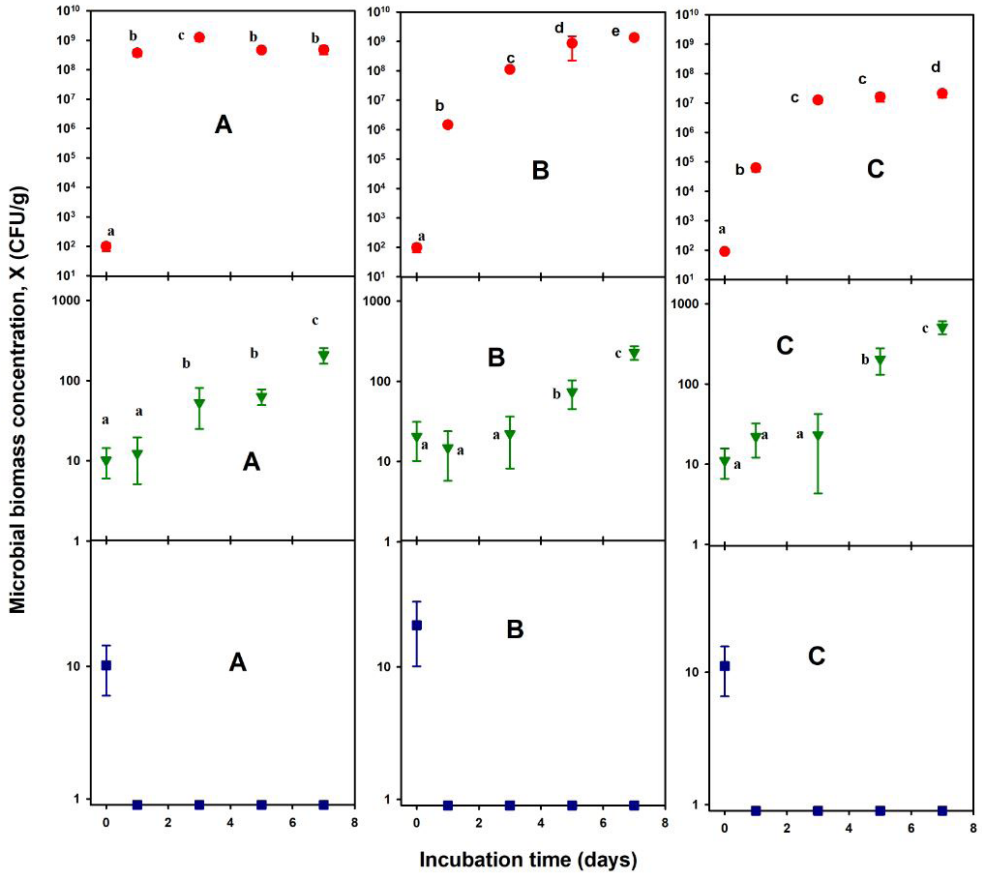

Figure 3. Growth kinetics of the tested bacteria (CFU/g) at 35 °C in a Mexican fresh cheese medium. (A) *Escherichia coli*; (B) *Staphylococcus aureus*, and (C) *Listeria monocytogenes*. Film treatments were: (■) F2 film (base films containing pectin and gellan gum, plus AMC, 90 AU/mL, and EDTA, 0.05 M); (▼) FC films (base films), and (●) bacterium inoculated culture media with no films. Runs were done by triplicate. Error bars are standard deviations, and same lower case letters indicate no statistical differences on the basis of Tukey's means comparison, P<0.05.

According to the former data, the moderate inhibition of the bacteria by FC films would be attributed to the antimicrobial effects of pectins within the films; nonetheless, the antimicrobial effectiveness would be influenced by the combination of diverse factors, including both physical and biochemical hurdles.

Moreover, an interesting antimicrobial activity was exhibited by the F2 films in MCh medium, which completely hindered the growth of the three tested bacteria during the experiments (Figure 3, square symbols) although they were inoculated in an enriched medium and incubated at optimal temperature (i.e., 35 °C). This bacterial growth inhibition would be due to a synergistic combination of the antimicrobial agents (i.e., AMC, EDTA and pectins) present in the active F2 films which resulted in an effective antimicrobial film. In an analogous study, Sivarooban et al. (2008) described a maximum antimicrobial activity of soy protein films added with grape seed extract (1% w/w), nisin (10,000 IU/g) and EDTA (0.16% w/w) which reduced the growth of *L. monocytogenes*, *E. coli* and *Salmonella typhimurium*. Also, Tajik et al. (2014) tested combinations of monolaurin and EDTA, from 250 to 2000 µg/mL, against *E. coli* and *S. aureus* in Iranian white cheese, obtaining reductions in the bacterial concentrations.

3.2 Mechanical characterization

Table 1 presents the selected physical-mechanical properties of both bioactive (F2) and control (FC) films, exhibiting parameters statistically different (P<0.001) between the two films. The addition of EDTA and AMC into the films increased the Young's modulus (EM), then F2 showed more resistance to axial deformation than FC. Hence, F2 was almost five times less extensible than FC, and the stress value at the end of the stretching of the FC was higher than that of the F2 film. The high stiffness of the bioactive film can be ascribed to the development of a heterogeneous film structure, probably due to the formation of crystals composed of calcium and H_2EDTA^{-2} ions and water molecules (Zabel et al., 2006), which could be formed during either drying or storage of the films. This effect was not expected; $CaCl_2$ was added to filmogenic solutions to promote gellan and pectin gelation and to yield biopolymer matrices with higher degree of cross-linking and therefore less gas-permeable films. High values of mechanical properties have been also reported for other bioactive films, for instance, pectin-polylactic acid-nisin films (EM = 2590 MPa, $\%E_{max}$ = 3%) (Jin et al., 2009), chitosan-eugenol and chitosan-cinnamon essential oil (EM = 1660 to 1460 MPa, $\%E_{max}$ = 6 to 8%) (Valencia-Sullca et al., 2016) and antimicrobial gelatin films reinforced with metallic nanoparticles (EM = 2.30 to 2.63 GPa, $\%E_{max}$ = 8.3 to 9%) (Shankar et al., 2016).

3.3 Water vapour permeability

The values of water vapour permeability, WVP, of both FC and F2 films are shown in Table 1, being statistically significant different between them (P<0.001). The WVP was affected by the incorporation of antimicrobial agents, mainly EDTA, then F2 film was near 85% less water-vapour permeable than FC. This decrease might be explained by structural modifications arose in the F2 network by the development of crystal aggregates of EDTA-calcium. This assumption is based on the reported observations in studies regarding films containing nanoparticles (i.e., nanoclays, nanofibers, nanowhiskers (Sanchez-Garcia et al., 2010). These studies have attributed the reduction in WVP of nanocomposite films to the high nanodispersion of the particles across the matrix, the high crystallinity and the good interfacial adhesion in the nanobiocomposites. However, it has been also reported that at high concentration of nanoparticles, the WVP increases due to the development of filler agglomeration, which usually yield the creation of preferential paths for the permeants to diffuse faster. Thus, the good dispersion of fillers into the biopolymer matrix as well as the amount and nature of the plasticizers could be relevant aspects to consider enhancing the water barrier properties of the films.

3.4 Oxygen permeability

The oxygen permeability (PO_2) of the films was not affected by the presence of EDTA and AMC (Table 1). There were not a statistically significant difference between the oxygen permeability of the two films (P=0.358). These values were lower than the corresponding WVP ones. The hydrophilic nature of the pectin-gellan films, their low plasticisation and their microstructural organization, provided a good barrier to oxygen diffusion and this could have enhanced the antimicrobial activity of these films, reported in previous sections.

4 Conclusion

Food-packaging films containing gellan gum, citric pectin, glycerol, $CaCl_2$, EDTA and antimicrobial activity-concentrated supernatant (AMC) from *Streptococcus infantarius*-fermentations, which contained bacteriocin-like inhibitory substances, were elaborated and characterized. The bioactive films exhibited interesting inhibitory effects on *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*, inoculated in both selective media

Table 1. Selected physical-mechanical properties of functional films. The control film (FC) contained 0.2% (w/v) gellan gum, 1% (w/v) citric pectin, 0.5% (w/v) glycerol and $CaCl_2$ 5 mM. F2 treatment: FC formula plus AMC, 90 AU/mL, and EDTA, 0.05 M. Mean values ± standard deviation.

Properties	FC	F2
Young's modulus (MPa)	707 ± 48 a	1,394 ± 144 b
Elongation at break (%)	9 ± 2 b	1.9 ± 0.4 a
Stress at break (MPa)	13 ± 1 b	5.7 ± 0.7 a
WVP (10^{-11} g m Pa^{-1} s^{-1} m^{-2})	19.8 ± 0.4 b	2.9 ± 0.8 a
PO_2 (10^{-12} g m Pa^{-1} s^{-1} m^{-2})	1.2 ± 0.2 a	2 ± 1 a

Values in the same row with different letters are statistically different ($p \leq 0.001$; t-test).

(MacConkey, Baird Parker and Oxford, respectively) and in a Mexican fresh cheese medium, where such antimicrobial activity would be attributed to an AMC-EDTA-Pectin synergy. On the other hand, the physical-mechanical properties of the films were influenced by the EDTA contents, yielding stronger and less extensible films than the control one; these effects might be attributed to the formation of EDTA-calcium crystals into the film biopolymer matrix. These crystals also enhanced the water barrier properties of the bioactive films. Besides, the films showed low oxygen permeabilities, which would contribute to their antimicrobial effectiveness.

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