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Physical, mechanical and antimicrobial properties of Argentine anchovy (*Engraulis anchoita*) protein films incorporated with organic acids

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ABSTRACT

Physical, mechanical and antimicrobial properties of protein films from Argentine anchovy (*Engraulis anchoita*) incorporated with 0, 0.50, 0.75 and 1.50% sorbic or benzoic acids were investigated against *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Enteritidis and *Staphylococcus aureus*. The effects of films containing 1.50% sorbic or benzoic acids were tested on meat inoculated with *Escherichia coli* O157:H7 and *Listeria monocytogenes* during storage at 5 °C. The increased concentration from 0.50 to 1.50% of sorbic and benzoic acids resulted in decreased tensile strength and increased elongation at break, color difference, opacity, water vapor permeability and solubility. Scanning electron microscopic images of the control film revealed a homogeneous and continuous structure and the presence of micropores on the film surface containing 1.50% of sorbic or benzoic acids, which contributed to the physical and mechanical properties of the resulting films. The control films did not inhibit many microorganisms tested in this study. Films with 1.50% sorbic or benzoic acids had the highest inhibition against *Escherichia coli* O157:H7, *Salmonella* Enteritidis and *Listeria monocytogenes*, however they did not inhibit *Staphylococcus aureus*. Films with 1.50% sorbic or benzoic acids applied in meat showed the greatest inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7. These results suggest that the films containing antimicrobials can be used to promote safety and quality of packaged meat, though the physical and mechanical properties of the films could be modified.

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1. Introduction

Recent food-borne microbial outbreaks are the driving force in seeking innovative ways to inhibit microbial growth in food while maintaining its quality and safety. Meat and meat products are the primary causes of food borne diseases and are also prone to spoilage during storage just as all proteinous foods (Cárdenas, Giannuzzi, & Zaritzkay, 2008; Emiroglu, Yemis, Coskun, & Candogan, 2010). Among these innovative strategies is the incorporation of antimicrobial agents in films, which can be used as active packaging (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Cagri, Ustunol, & Ryser, 2004; Emiroglu et al., 2010; Guillard, Issoupov, Redl, & Gontard, 2009; Zinoviadou, Koutsoumanis, & Biliaderis, 2010). Many studies have demonstrated that, when incorporated into edible films and coatings, antimicrobial agents can be effective in reducing levels of pathogenic organisms like *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhi and *Staphylococcus aureus* (Ahmad et al., 2012; Cagri, Ustunol, & Ryser, 2001; Kristo, Koutsoumanis, & Biliaderis, 2008; Ojagh, Rezaei, Razavi, & Hosseini, 2010; Ramos et al., 2012a).

Different matrices can be used to incorporate antimicrobial agents (Cagri et al., 2001, 2004; Ojagh et al., 2010; Tongnuanchan, Benjakul, & Prodpran, 2012). Fish proteins including myofibrillar and sarcoplasmic proteins have been used as film-forming materials (García & Sobral, 2005; Rocha, Loiko, Gautério, Tondo, & Prentice, 2013; Shiku, Hamaguchi, Benjakul, Visessanguan, & Tanaka, 2004). In Brazil, the Argentine anchovy (*Engraulis anchoita*) is an unexploited pelagic fish species (Castello & Castello, 2003), which raises the interest in obtaining proteins used for the preparation of films. Rocha et al. (2013) elaborated films based on Argentine anchovy protein isolate with good characteristics. Proteins have been extensively used because of their relative abundance, film-forming ability and nutritional qualities (Jiang, Xiong, Newman, & Rentfrow, 2012; Min, Rumsey, & Krochta, 2008; Ramos et al., 2013; Rocha et al., 2013). In general, the resistance to water vapor transmission of protein films is limited because they are highly polar polymers with a high level of hydrogen bonding and hydroxyl groups. The mechanical properties of protein-based

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films are better than those of lipids and polysaccharides. The proteins have a structure based on 20 different monomers conferring a wider range of functional properties, resulting in an elevated intermolecular binding potential which can form bonds at different positions (Kokoszka, Debeaufort, Lenart, & Voilley, 2010; Rocha et al., 2013). Even though the addition of antimicrobials and the method of film formation can alter the mechanical properties of proteins films, the antimicrobial and physical properties of protein films with various antimicrobials have been discussed (Ojagh et al., 2010; Sayanjali, Ghanbarzadeh, & Ghiassifar, 2011).

The addition of antimicrobial agents in films may enable the extension of the shelf-life and increase safety of packaged food, inhibiting the growth of spoilage or pathogenic microorganisms on the food surface (Emiroglu et al., 2010; Ojagh et al., 2010; Salgado, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2013; Tongnuanchan et al., 2012; Zinoviadou et al., 2010). Moreover, their relatively low rates of diffusion from the packaging material into the product collaborated with keeping the concentration of the active ingredient relatively high on food surfaces (Kristo et al., 2008). The most frequent antimicrobials incorporated in food packaging films are organic acids (e.g. sorbic, benzoic, citric and propionic acids), enzymes (e.g. lysozyme), bacteriocins (e.g. nisin), polysaccharides (e.g. chitosan), essential oils (e.g. bergamot) among others (Ahmad et al., 2012; Cagri et al., 2001, 2004; Min et al., 2008; Ramos, Silva, et al., 2012; Salgado et al., 2013). Zinoviadou et al. (2010) showed that whey protein isolate based films incorporated with sodium lactate (NaL) and ε-polylysine (ε-PL), when utilized as active packaging, presented an effect against spoilage flora of fresh beef. In their study, Salgado et al. (2013) verified that sunflower protein films incorporated with clove essential oil and applied to the preservation of refrigerated sardine patties inhibited the multiplication of total mesophiles.

Organic acids have a long history as GRAS (generally recognized as safe) food preservatives by U.S. Food and Drug Administration (Cagri et al., 2001, 2004; Guillard et al., 2009). Sorbic and benzoic acids and their salts are active against yeast, molds and many bacterium (Guillard et al., 2009). Cagri et al. (2001) found that whey protein based edible films, incorporated with sorbic acid inhibited the proliferation of *L. monocytogenes* and *E. coli* O157:H7. According to Manab, Sawitri, Alawwaly, and Purnomo (2011), the benzoic acid incorporated in whey protein based films significantly inhibited *E. coli* and *Salmonella* sp. According to Mani-López, García, and López-Malo (2011) the organic acids affect the microbial activity by cytoplasmic acidification with subsequent uncoupling of energy production and regulation and by accumulation of the dissociated acid anion to toxic levels.

Antimicrobial films must have adequate physical properties. Thus, integrated analyzes of antimicrobial, physical and mechanical properties are crucial for developing films (Ahmad et al., 2012; Cagri et al., 2001; Min et al., 2008). Therefore, this study aimed at (1) analyzing the properties, including thickness, color difference, opacity, tensile strength, elongation at break, solubility in water and water vapor permeability of antimicrobial protein films from Argentine anchovy (*E. anchoita*) in order to (2) measure the antimicrobial activity of films incorporated with sorbic or benzoic acids against *E. coli* O157:H7, *L. monocytogenes*, *S.* Enteritidis and *S. aureus*, as well as (3) assess the effectiveness of the films against the *L. monocytogenes* and *E. coli* O157:H7 inoculated in meat during storage at 5 °C.

2. Materials and methods

2.1. Materials

Argentine anchovy (*E. anchoita*) protein isolate (API) was obtained in the Laboratory of Food Technology at the Federal University of Rio Grande (FURG) using pH-shifting process according to Nolsoe and Undeland (2009) and Rocha et al. (2013). The mechanically separated meat (MSM) of anchovy was homogenized with distilled water in a ratio of 1:9 (MSM: water, w/v). Alkaline solubilization was performed under pH 11.2 (1 M NaOH) for 20 min at 4 °C. After solubilization, the sample was centrifuged at 9000 × *g* for 20 min. The soluble proteins were subjected to isoelectric precipitation carried out at pH 5.0 (1 M HCl) for 20 min at 4 °C and centrifuged at 9000 × *g* for 20 min. The precipitated protein was dehydrated in an air circulation oven (Quimis, Q342, São Paulo, Brazil) for 16 h at 40 °C. The Argentine anchovy protein isolate was analyzed in triplicate to determine the protein content, using classical methods by AOAC (2000) and the same presented a protein content of 88.8%.

The commercial samples of benzoic and sorbic acids were provided by Synth (São Paulo, Brazil). Used as plasticizer, glycerol was purchased from Synth (São Paulo, Brazil). The bovine meat cut (*knuckle*) was bought from a local market in Porto Alegre, Southern Brazil. The samples were transferred to the Laboratory of Food Microbiology of the Institute of Food Science and Technology of Federal University of Rio Grande do Sul (UFRGS) in thermal boxes and stored at 4 °C for 2 h before preparation. The meat samples were previously analyzed to prove the absence of initial contamination with psychrotrophic and mesophilic microorganisms.

2.2. Microorganisms

The bacteria used in this study were *E. coli* O157:H7, *S. aureus* (ATCC 25953), *S.* Enteritidis (SE86) and *L. monocytogenes*. They were transferred from the Culture Collection of the Laboratory of Food Microbiology of Institute of Food Science and Technology at the Federal University of Rio Grande do Sul (ICTA/UFRGS).

2.3. Film preparation

Antimicrobial films were prepared following the method adapted by Cagri et al. (2001) and Rocha et al. (2013). The film forming solution (FFS) was prepared by mixing 3.0 g of API and 100 mL distilled water, under continuous stirring at 500 rpm in a mechanical stirrer (Fisatom, 712, São Paulo, Brazil) at 25 °C for 25 min. The pH was adjusted to 11.5 with 1.0 M NaOH, and glycerol (30 g of glycerol/100 g of API) was added as a plasticizer. The mixture was homogenized with an Ultra-turrax (IKA, T25, Werke, Germany) at 10.000 rpm for 10 min. The FFS was placed in a glass reactor coupled in an ultrathermostatic water bath at 74 °C for 30 min and stirred continuously in order to form a film matrix through denaturation of protein, then filtered by cheesecloth and cooled to 23 ± 2 °C. The FFS that did not contain antimicrobial agents, served as control. Following, sorbic acid (SA) or benzoic acid (BA), were incorporated in the FFS 0.50%, 0.75% or 1.50% (w/v). The pH was adjusted to 5.2 using 1.0 M HCl. FFS (25 mL) was put on Petri dishes (9 cm in diameter) and dried at 35 °C for 16 h, after which the films were peeled from the plates and conditioned in a desiccator containing silica at 25 °C for 48 h prior to testing.

2.4. Properties of films

2.4.1. Film thickness

Film thickness (mm) was determined using a digital micrometer (IP65, Insize, São Paulo, Brazil) having a precision of 0.001 mm. Ten random locations around each film sample were used to determine thickness, and the mean value was used to calculate water vapor permeability and mechanical properties.

2.4.2. Color and opacity

Film color was measured using a CR-400 Minolta Chroma Meter (Minolta, CR-400, Osaka, Japan). The instrument was calibrated with a standard white plate, and the *CIE Lab* color space was used (CIE, 1986). The color of the films was expressed as total difference in color (ΔE^*) obtained using illuminant D_{65}. Three measurements were taken from each sample. Total difference in color (ΔE^*) was calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard ($L = 97.39$, $a^* = -0.14$, $b^* = 1.94$). Where L^* (lightness/brightness), a^* (redness/greenness) and b^* (yellowness/blueness) values.

Film opacity was determined with the same equipment used for color measure, also operating in the reflectance mode. The opacity (Y) of the samples was calculated as the relationship among the opacity of each sample on the black standard (Y_b) and the opacity of each sample on the white standard (Y_w). Each type of film was prepared and evaluated in triplicate.

2.4.3. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of films were determined according to the ASTM Standard Method D882-91 (ASTM, 1996) using a Texture Analyzer (TA.XT$_{plus}$, Stable Micro Systems, England). Films were cut into 25 mm by 80 mm strips, initial grip separation and cross-head speed were set at 50 mm and 0.8 mm/s, respectively (Rocha et al., 2013). TS (MPa) was calculated by dividing the maximum force by the initial specimen cross-sectional area of the film, while EAB (%) was calculated by dividing film elongation at break by the initial gauge length of the specimen and multiplying by 100. Each film type was prepared and assessed in triplicate.

2.4.4. Film solubility

The solubility (%) of the film samples in water was measured according to the method by Gontard, Duchez, Cuq, and Guilbert (1994) with modification. Small films (2 cm in diameter) were dried in an oven (DeLeo A1 SED, Porto Alegre, Brazil) at 105 °C at constant weight, to determine the initial dry weight. Films were immersed into 50 mL of distilled water and the mixtures were shaken continuously at 100 rpm at 25 °C for 24 h using a shaker (Cientec, CT-712RNT, Piracicaba, Brazil). After immersion, the films were dried at 105 °C at constant weight (final dry weight). Film solubility (%) was defined as the ratio between the water-soluble solid content and initial solid dry content (× 100). Each film type was prepared and assessed in triplicate.

2.4.5. Water vapor permeability (WVP)

WVP was determined gravimetrically at 25 °C according to the method described by ASTM Standard Method E96–95 (ASTM, 1995) with modifications. The films were sealed on a permeation cell (exposed area: 1.58×10^{-3} m^2) containing anhydrous calcium chloride (0% RH). The cell was placed in desiccators with a saturated sodium chloride solution (75% RH). The cells were weighed at 24 h intervals for 48 h at 25 °C. Each film type was prepared and assessed in triplicate. The Film WVP (g mm/m^2 d kPa) was calculated as follows:

$$\mathrm{WVP} = \frac{w\,L}{A t\,\Delta P}$$

where w is the weight gain of the permeation cell (g); L is the film thickness (mm); A is the exposed area of film (m^2); t is the time of weight gain (days); ΔP is the vapor pressure difference across the film (kPa).

2.4.6. Scanning electron microscopy (SEM)

In order to study the structure of the API films with or without incorporation of SA or BA and then assess their homogeneity, some SEM experiments were carried out. The microstructural characteristics of the films surface were examined using a scanning electron microscope (Jeol, JSM-6060, Japan) at an accelerating voltage of 10 kV. Prior to visualization, the film samples were mounted on aluminum stub and sputtered with gold (Sputter Coater, SCDO50) to make the sample conductive. Film samples were observed at the 2000× magnification.

2.5. Antimicrobial activity

Antimicrobial activity testing of the API films incorporated with different concentrations (0.50%, 0.75% and 1.50%) of SA or BA and control films (without organic acids) was carried out by the inhibition zone test according to CLSI (2003). The zone of inhibition on solid media was used for determining antimicrobial effects of films against typical food pathogens including *E. coli* O157:H7, *S.* Enteritidis, *L. monocytogenes* and *S. aureus*. The microorganism used in the microbiological assay remained in the nutrient agar (Oxoid Ltd., Hampshire, England) culture for 24 h. From the nutrient agar, the colonies were inoculated into 5 mL of 0.85% saline solution (Oxoid Ltd., Hampshire, England) to obtain a turbidity standard corresponding to the number 0.5 of the McFarland scale, which represents a total of $1–2 \times 10^8$ CFU/mL. The films samples were aseptically cut into 15 mm diameter discs using a circular knife, then placed on MH agar plates, which had been previously smeared with a sterile swab dipped into the suspension with inoculums ($1–2 \times 10^8$ CFU/mL) and spread over the surface of the Mueller-Hinton (MH) agar plate (Oxoid Ltd., Hampshire, England). Afterwards, the plates were incubated inverted at 37 °C for 24 h and the diameters of inhibition zones around the film disc were measured in triplicate.

2.6. Application of films on meat

Application of films on meat was prepared according to the method used by Emiroglu et al. (2010) and Zinoviadou et al. (2010) with modifications. Based on the results obtained from the antibacterial activity test and considering a possible diffusion of antimicrobial agents from the film matrix into meat, the films were used to evaluate antimicrobial activity on the meat inoculated with the microorganisms inhibited by SA or BA. Control samples were packed in polyethylene films. The meat was divided in small pieces ($2.1 \times 2.5 \times 1.0$ cm) of 5.0 g (± 2.0) using a stainless steel knife. The chemical composition of beef was as follows: 70.2% (± 0.5) humidity, 22.3% (± 0.7) proteins, 6.6% (± 0.2) lipids, and 1.1% (± 0.1) ashes. Chemical composition was determined using AOAC (2000) standard methods. The samples were immersed in 0.1% sterile peptone water (w/v) (Oxoid Ltd., Hampshire, England) containing approximately 4.7×10^5 and 4.9×10^4 CFU/mL the inoculum to *L. monocytogenes* and *E. coli* O157:H7, respectively, and homogenized at room temperature for 1 min. These were wrapped in cross-shaped antimicrobial films that covered the entire meat surface. The meat samples were placed into sterile Petri dishes and stored at 5 °C (± 1 °C). All samples were evaluated periodically for microbiological quality (0, 2, 4, 6, 8, 10 and 12 days).

2.6.1. Microbiological analysis

Throughout the storage of the meat cuts, samples were taken and analyzed. Meat samples (5.0 ± 2.0 g) with the surrounding

films were aseptically removed from the Petri dishes, added into 45 mL of 0.1% sterile peptone water (w/v) (Oxoid Ltd., Hampshire, England) and homogenized in a stomacher (Seward, USA) for 2 min at room temperature. The film was removed and added together with the meat sample to wash off the bacteria that could be attached on its surface. The counts of microorganisms were performed by the plating method in drops according to Silva et al. (2001) with adaptations. Aliquots of 30 μL of appropriate serial dilutions from 10^{-1} to 10^{-7} were spread plated on brain heart infusion (BHI) agar (Oxoid Ltd., Hampshire, England) and BHI agar containing 0.6% Yeast Extract (Oxoid Ltd., Hampshire, England) for *E. coli* O157:H7 and *L. monocytogenes*, respectively. The plates were inversely incubated at 37 °C for 24 h. The results were expressed as means of triplicate in log CFU/g.

3. Results and discussion

3.1. Properties of the films

The Argentine anchovy (*E. anchoita*) protein films incorporated or not with different concentrations of sorbic or benzoic acids were visually homogeneous and flexible.

3.1.1. Thickness, color and opacity

Film thickness is an important factor for the optimal functional performance (Akhtar et al., 2010). Thickness values of API films incorporated with SA or BA are shown in Table 1. The control film showed a thickness of 0.150 mm, this result was lower than those found for films based on protein isolate from Argentine anchovy, which showed a thickness of 0.172 mm (Rocha et al., 2013). The increase of concentration of organic acids from 0 to 1.50% in films increased the film thickness ($p < 0.05$), resulting in a range of values from 0.150 to 0.185 mm and 0.150 to 0.217 mm for films incorporated with SA and BA respectively. Cagri et al. (2001) showed that whey protein films showed an increase in thickness when the content of SA added into a FFS was increased from 0.50 to 1.50% (w/v). According to Akhtar et al. (2010) thickness is highly dependent on the concentration of dry matter and film preparation methods. In this study, the increase in thickness occurs due to the raise of antimicrobial agent content in FFS.

According to some authors (Akhtar et al., 2010; Ghasemlou, Khodaiyan, & Oromiehie, 2011) the parameters of color and opacity are important in terms of general appearance, consumer acceptance and in food packaging applications. Color difference (ΔE^*) and opacity of films were shown in Fig. 1. The addition of antimicrobial agents in FFS significantly reduced ($p < 0.05$) the transparency of the films, because they blocked the passage of light through the film, increasing the opacity. These results suggested that incorporation of SA or BA had an influence in film color, but changes depend on the type of antimicrobial agents. In this study films incorporated with SA were more opaque and dark, when compared to the other films produced ($p < 0.05$). According to Flores, Haedo, Campos, and Gerschenson (2007) the increase of sorbate content and sorbic acid salt, increased the ΔE^* and reduced the degree of transparency of the film of tapioca starch. However, Jipa, Stoica-Guzun, and Stroescu (2012) found that the incorporation of SA in PVA films resulted in films with lower ΔE^* due to a significant increase in the rate of white and yellowness index decreased as a result of the formation of crystals, which was not observed in this study. Ramos, Santos, et al. (2012) developed films based on protein isolate whey incorporated with lactic and propionic acids with color difference ranging from 6.4 to 9.4. These films were brighter than films elaborated with API incorporated with the organic acids as showed in Fig. 1. According Rocha et al. (2013) the protein isolate obtained from Argentine anchovy, which has a dark muscle, resulted in an increase of color in the film. According to Chaijan, Benjakul, Visessanguan, and Faustman (2004) dark muscle contains an elevated content of myoglobin than ordinary muscle and those pigments can contribute to the darker color of films compared with films from other muscles.

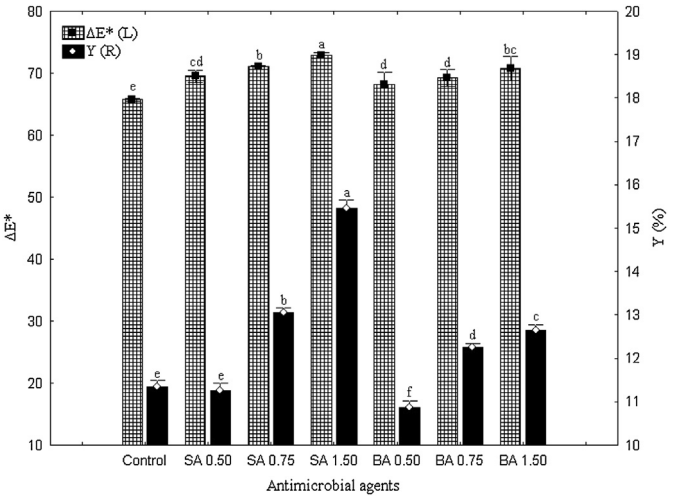

Fig. 1. Color difference – ΔE^* (left) and opacity – Y (right) of different films elaborated with various concentrations (0, 0.50, 0.75 and 1.50%) of sorbic acid (SA) or benzoic acid (BA). Different letters in different column for the same parameter tested indicate significant differences between type or concentration of antimicrobial agent ($p < 0.05$).

3.1.2. Mechanical properties

The films are expected to have adequate strength, elongation at break and maintain constant integrity on food products during handling and storage (Ghasemlou et al., 2011). The interaction of protein, hydrocolloids and other additives including water, plasticizers, with antimicrobial agents determine the mechanical properties of the film. Tensile strength (TS) and elongation at break

Table 1
Thickness, solubility, water vapor permeability (WVP), tensile strength (TS) and elongation (EAB) of anchovy protein films incorporated with sorbic acid (SA) or benzoic acid (BA) at various concentrations.

Film	Thickness (mm)	Solubility (%)	WVP (g mm/m^2 d kPa)	TS (MPa)	EAB (%)
Control 0%	0.150 ± 0.050^{d}	27.70 ± 1.47^{f}	8.41 ± 0.10^{d}	3.90 ± 0.09^{a}	9.00 ± 1.00^{f}
SA 0.50%	0.156 ± 0.001^{d}	31.58 ± 0.47^{e}	14.95 ± 0.88^{c}	2.62 ± 0.01^{b}	28.30 ± 0.60^{e}
SA 0.75%	0.172 ± 0.001^{c}	35.04 ± 0.84^{d}	15.33 ± 0.58^{c}	1.40 ± 0.01^{d}	43.20 ± 0.35^{d}
SA 1.50%	0.185 ± 0.010^{c}	65.34 ± 0.88^{a}	19.30 ± 0.95^{ab}	1.11 ± 0.11^{d}	121.33 ± 2.65^{a}
BA 0.50%	0.150 ± 0.010^{d}	34.84 ± 0.53^{d}	15.95 ± 0.60^{c}	3.80 ± 0.22^{a}	28.70 ± 1.51^{e}
BA 0.75%	0.200 ± 0.001^{b}	39.92 ± 0.48^{c}	19.14 ± 0.55^{b}	2.00 ± 0.01^{c}	57.97 ± 0.11^{c}
BA 1.50%	0.217 ± 0.001^{a}	46.74 ± 0.58^{b}	21.04 ± 0.92^{a}	1.21 ± 0.18^{d}	97.66 ± 2.08^{b}

* The mean ± standard error (n = 3). Means in same column with different superscript are significantly different ($p < 0.05$).

(EAB) of protein isolate films incorporated with various concentrations of SA and BA are shown in Table 1. The control film showed higher TS (3.90 MPa) and lower EAB (9.00%) than films incorporated with SA or BA. When SA and BA concentrations were increased from 0.50% to 1.50%, EAB also increased from 28.30% to 121.33% and from 28.70% to 97.66% ($p < 0.05$) respectively. On the other hand, TS of antimicrobial films significantly decreased when compared with the control film ($p < 0.05$) from 2.62 MPa to 1.11 MPa and from 3.80 MPa to 1.21 MPa with increasing content of SA and BA from 0.50 to 1.50%, respectively. Increasing the content of additives other than cross-linking agents resulted in films with lower TS and better EAB, because these molecules can be inserted between protein chains to form hydrogen bonds with amide groups of proteins. Therefore, reduced interactions between these protein chains increase flexibility (Guilbert, 1986). Cagri et al. (2001) showed that when the SA concentrations increased from 0.50% to 1.50% in films based on whey protein isolate (WPI), the EAB increased from 20.00% to 74.28%. While TS of WPI films decreased from 4.85 MPa to 3.05 MPa with increasing content of SA. Kristo et al. (2008) verified that films based on sodium caseinate with an increase in potassium sorbate content incorporated from 10% to 25% resulted in reduction of TS and increase in EAB. According to them the potassium sorbate also acted as plasticizer, since increasing amounts of potassium sorbate resulted in reduction of TS and increase in of EAB. According to Sayanjali et al. (2011) the addition of potassium sorbate as an antimicrobial component in polymeric matrix of carboxymethylcellulose films causes a weakening in the structure resulting in an increase in the elongation at break and reduction in tensile strength. However, in their studies these authors verified that the incorporation of potassium sorbate up to 1 g, in carboxymethyl cellulose films plasticized with glycerol, significantly increased EAB values ($p < 0.05$), while an incorporation of 4 g of sorbate, decreased elongation at break. This reduction showed that higher levels of sorbate weakened the polymer structure and thereby decreasing the EAB value.

In this study, films containing SA had lower TS and higher EAB when compared to films containing BA. According to Cagri et al. (2001), the straight chain of SA can penetrate into protein isolate chains more easily than BA, which has an aromatic ring like benzene, forming hydrogen bonds with amide groups of proteins. Consequently, the SA allows greater mobility between protein chains, thereby producing films of lower TS and greater flexibility. Affected by the incorporation of additives, the effect in mechanical properties has been investigated previously in several hydrocolloid-based films (Ahmad et al., 2012; Kristo et al., 2008; Ramos, Silva, et al., 2012; Salgado et al., 2013; Sayanjali et al., 2011; Tongnuanchan et al., 2012).

3.1.3. Water vapor permeability and solubility

Water vapor permeability (WVP) and solubility are important factors related to the future applications of films. WVP and solubility results are demonstrated in Table 1. Films based on proteins tend to be permeable due to the high number of polar amino acids in their constitution, which contribute to the affinity between film and water vapor (García & Sobral, 2005; Jiang et al., 2012; Tongnuanchan, Benjakul, & Prodpran, 2011). The control film showed lower WVP (8.41 g mm/m^{2}d kPa) when compared with films incorporated with SA or BA. The antimicrobial agents contributed to extend intermolecular interaction and furthermore, loosening the compactness of the structure. The moisture then passing through the enhanced films and thereby WVP values of the films increased (Pranoto, Rakshit, & Salokhe, 2005). According to Rocha et al. (2013) the polar amino acids correspond to about 53.3% of the total amino acids in the API. Furthermore, the incorporation of organic acids increased WVP. The increase in the amount of SA and BA from 0.50% to 1.50% raised WVP ($p < 0.05$) from 14.95 g mm/m^{2}d kPa to 19.30 g mm/m^2 d kPa and from 15.95 g mm/m^2 d kPa to 21.04 g mm/m^2 d kPa, respectively. Cagri et al. (2001) in their study verified that films based on whey protein isolate without SA showed a WVP of 27.24 g mm/m^{2}d kPa. According to Sayanjali et al. (2011) films based on carboxymethyl cellulose containing potassium sorbate (2 g/100 mL) showed a water vapor permeability of 15.5 g mm/m^{2}d kPa.

According to Kristo et al. (2008) increasing of potassium sorbate content in films based on sodium caseinate implies a plasticizing action which showed an increase of WVP. The same authors verified that the addition of potassium sorbate into sodium caseinate films which possessed the ability to absorb water contributed to the augmentation of the diffusion constant of water vapor and hence the WVP. Cagri et al. (2001) found an increase in WVP of whey protein film from 27.25 g mm/m^2 d kPa to 43.76 g mm/m^2 d kPa with the addition of SA content from 0.50% to 1.50%. Such results suggested that WVP in films also depends on the hydrophilic-hydrophobic ratio of the film constituents, such as the type of protein, plasticizer and antimicrobial agent (Ahmad et al., 2012). Although protein films are excellent oxygen barriers, generally they are poorly resistant to moisture permeation (Jiang et al., 2012).

The solubility of both SA and BA films increased when the concentration of antimicrobials was higher ($p < 0.05$). The control films showed 27.70% water solubility, this is significantly less than water soluble films with the incorporation of various organic acids. The values of water solubility of the films incorporated with SA and BA ranged from 31.58% to 65.34% and from 34.84% to 46.74% with an increase in the content of organic acids from 0.50% to 1.50% respectively. The obtained values for solubility are slightly higher than those observed in whey protein based films incorporated with propionic acid and BA (Manab, Sawitri, & Zanah, 2007). According to Baird-Parker (1980) the short chain of organic acids such as SA and BA contributes to high solubility, which was verified in this study, when the addition of SA and BA resulted in films with greater solubility (65.34% and 46.74%) compared to other films made with even lower content of such organic acids. In addition, the non-crosslinked proteins are expected to form hydrogen bonds with -OH groups of water molecules, thus increasing the susceptibility to hydration and so leading to increases in moisture content, solubility and permeability. This shows that the hydrophilicity of the additives considered will increase the water content of the film, which would thus affect the solubility therein (Sobral, Menegalli, Hubinger, & Roques, 2001). Although higher solubility indicated lower water resistance, high solubility may be an advantage for some applications. Additionally, the dissolution of film, an edible packaging, provides the advantage for application, since the removal of the film is not required before cooking or consumption (Ahmad et al., 2012).

3.1.4. Scanning electron microscopy (SEM)

SEM may be used to evaluate film homogeneity, layer structure, pores, cracks and surface smoothness. The permeability of films can be influenced by composition, morphology and homogeneity of the film matrix (Bilbao-Sainz, Avena-Bustillos, Wood, Williams, & McHugh, 2010). The effect of incorporation of higher content of SA and BA on the surface microstructure is shown in Fig. 2. The microstructure showed in the control film (Fig. 2a) revealed a homogeneous and continuous structure. SEM revealed that the structure of SA (1.50%) film was homogeneous and showed little uniformly distributed pores throughout the film (Fig. 2b). The film containing BA (1.50%, Fig. 2c) had a rough surface, which provides ease of permeation, resulting in an increase in WVP.

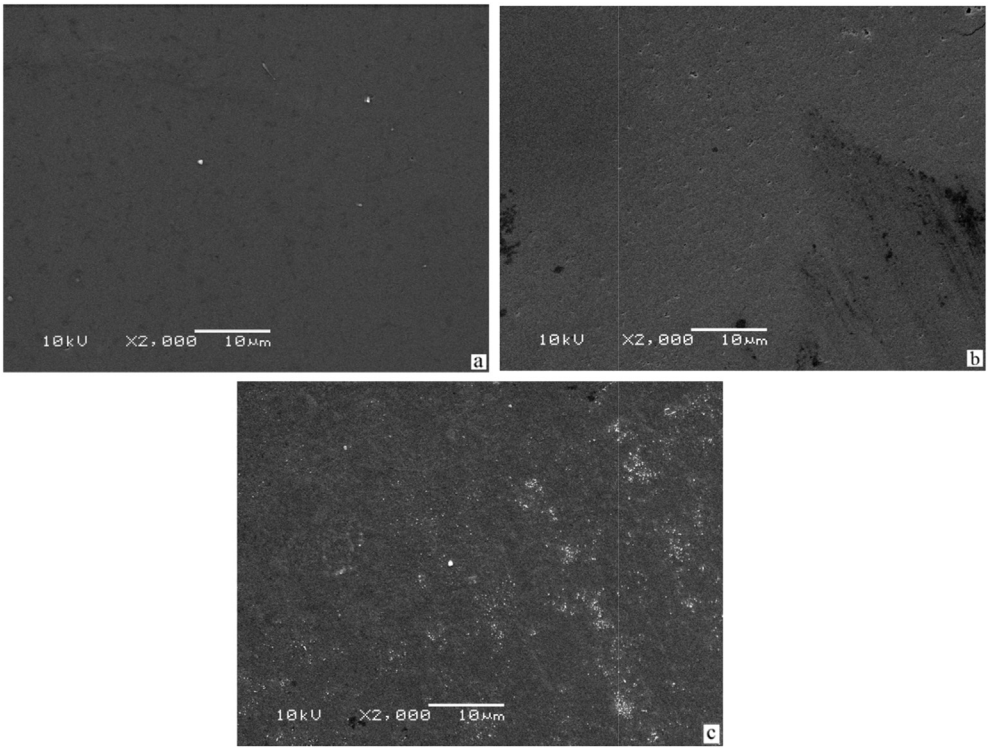

Fig. 2. SEM micrographs of the surface of the anchovy protein films incorporated with antimicrobial agents. (a) Control films; (b) 1.50% SA and (c) 1.50% BA.

3.2. Antimicrobial activity

The antimicrobial activities of API films incorporated with SA or BA against *E. coli* O157:H7, *S.* Enteritidis, *L. monocytogenes* and *S. aureus* are shown in Fig. 3. The film without SA or BA was used as a means of control to determine any possible antimicrobial effect of the film without additives. According to the results, the control film did not demonstrate inhibitory effect in any of the microorganisms tested. On the other hand, the films showed antimicrobial effect against *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* after the incorporation of SA or BA. According to Pérez, Balagué, Rubioloc, and Verdinia (2011), control films without addition of antimicrobial agents did not show inhibitory effect in *E. coli*. In this study, the size of the inhibition zones increased significantly when the concentration of SA or BA increased ($p < 0.05$).

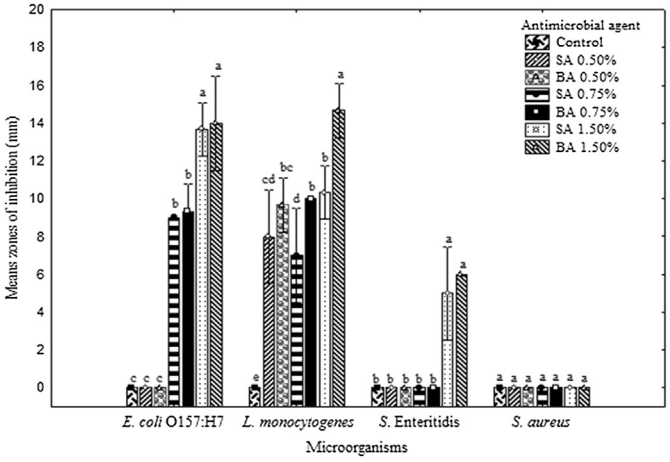

Fig. 3. Inhibition zone diameters by anchovy protein based film disks with various concentrations (0, 0.50, 0.75 and 1.50%) of sorbic acid (SA) or benzoic acid (BA) against test organisms. Different letters in different column for the same microorganism tested indicate significant differences between type or concentration of antimicrobial agent ($p < 0.05$).

In this study, *L. monocytogenes* was inhibited by low SA and BA content, shown in Fig. 3. The other three pathogens were not inhibited by the same concentration of organic acids. These results agree with Holley and Patel (2005) who reported that Gram-positive bacteria are, in general, more sensitive to organic acids than Gram-negative. *L. monocytogenes* was inhibited when using films containing BA or SA at levels from 0.50% to 1.50% with inhibition zones ranging from 9.67 mm to 14.67 mm and 8.00 mm to 10.34 mm, respectively. These differences in inhibition zone might be due to the intrinsic properties of the antimicrobial agents at the concentrations used and the rate of diffusion of the antimicrobials. Cagri et al. (2001) also reported inhibition against *L. monocytogenes* when SA was used at levels of 0.50%, 0.75%, 1.00% and 1.50% with inhibition zones ranging from 12.30 mm to 32.00 mm. The organic acids are considered to affect microbial activity by two primary mechanisms: cytoplasmic acidification with subsequent uncoupling of energy production and regulation, and accumulation of the dissociated acid anion to toxic levels (Mani-López et al., 2011).

Films containing 1.50% of SA or BA were the most effective against *E. coli* O157:H7, which showed the largest inhibition zone of 13.00 mm and 14.34 mm respectively ($p < 0.05$). Meanwhile, 0.75% of the same antimicrobials showed inhibition zones of 9.00 mm and 9.34 mm respectively. Cagri et al. (2001) showed lower inhibition than that found in this study for *E. coli* O157:H7 using films incorporated with SA. *S.* Enteritidis was the most resistant to API films containing 0.50% and 0.75% with SA or BA. Manab et al. (2011) showed that *Salmonella* sp. was the most resistant microorganism to whey protein based films containing acetic acid, lactic acid, propionic acid and benzoic acid, and this is possibly due to the adaptation capacity of such microorganism against organic acids.

Salmonella specifically has the ability to adjust to acid environments and survive in drastic pH conditions. *Salmonella's* mechanism for acid resistance has been established and is fairly complex (Mani-López et al., 2011). However, films incorporated with 1.50% of SA or BA had inhibition zones of 5.00 mm and 6.00 mm respectively. These results suggest that higher content of SA or BA is required to inhibit the growth of *S.* Enteritidis.

Despite being a Gram-positive microorganism, *S. aureus* showed no inhibition at different levels and types of antimicrobial agents tested in this study. Park et al. (2001) investigated the antimicrobial activity of polymers containing phenol and benzoic acid derivatives against *S. aureus* and others microorganisms. They showed that higher content of benzoic acid derivatives are required for *S. aureus* inhibition. These results could be a consequence of the difference between organic acids effectiveness against the microorganisms tested. Organic acids and their salts are considered weak acids, meaning they do not fully dissociate in water but do so in a pH-dependent manner. As a result, the antimicrobial activity of organic acids is enhanced as the pH of the food is lowered to, or below, the pKa of the acid due to their increased ability to penetrate the cytoplasmic membrane of bacteria. The pKa is defined as the acid dissociation constant (Mani-López et al., 2011). According to Cagri et al. (2001) at pH 5.2, 28.48% of SA (pKa = 4.75) is undissociated. At pH 5.0 approximately 12.8% of BA is in the undissociated form. Chen, Yeh, and Chiang (1996) measured the diffusion rate of SA and BA incorporated in chitosan films where 57% of AS and 65% of BA were released by the films. Furthermore, the result of disk diffusion test is known to be dependent on many factors, including size, shape, and polarity of diffusants and chemical structure and level of cross-linking of diffusion media (Cagri et al., 2001). The different interactions of the films based on API with BA or SA may result in diffusion rates, levels of inhibition and different results.

3.3. Application of films on meat

Films incorporated with the highest content (1.50%) of SA and BA were more effective in inhibiting *E. coli* O157:H7 and *L. monocytogenes*. Therefore, these films were used as active packaging of the meat inoculated with these organisms to evaluate their antimicrobial effect on the surface of the beef. The beef sample showed no significant psychrotrophic and mesophilic counts, suggesting that they were manipulated hygienically and sanitarily.

The effect of antimicrobial films on the growth of *E. coli* O157:H7 and *L. monocytogenes* on meat is shown in Fig. 4. As it can be seen on the graph there were significant differences ($p < 0.05$) only between microorganisms in day zero of the storage, because the initial concentrations of *L. monocytogenes* and *E. coli* O157:H7 on inoculated meat samples were 5.7 log CFU/mL and 4.6 log CFU/mL respectively. There was a significant increase ($p < 0.05$) in growth of *L. monocytogenes* and *E. coli* O157:H7 on control films from 0 to 12 days of storage, compared to the other samples packed with antimicrobial films. After 2 days of storage at 5 °C, there was no significant growth ($p > 0.05$) of *L. monocytogenes* on meat samples covered with SA or BA films. Nevertheless, the film containing BA showed greater inhibition ($p < 0.05$) than the film containing SA against *E. coli* O157:H7. According to Harris and Thorarensen (2004) the antimicrobial agents can act on the bacteria by the interruption of its multiplication, exerting a bacteriostatic effect. Such behavior was observed in this study from days 2 to 6 of storage when the multiplication of *E. coli* O157: H7 was similar to the samples packaged with SA. The multiplication from days 2 to 6 of *L. monocytogenes* was reduced from 5.6 log CFU/g to 4.6 log CFU/g and from 5.6 log CFU/g to 4.6 log CFU/g respectively, with films containing BA and SA. According to Ramos, Santos, et al. (2012) the antimicrobial resistance mechanisms in Gram-negative bacteria are more complicated than those present in Gram-positive bacteria, once the former has an outer characteristic membrane, which acts as an extra barrier to the action of organic acids on the cytoplasmic membrane.

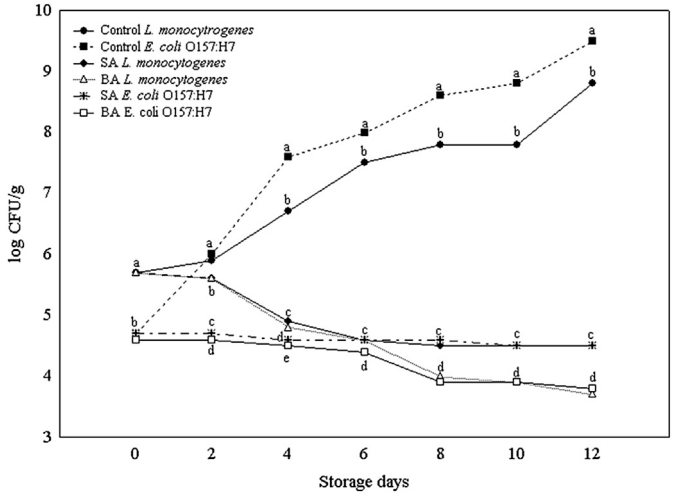

Fig. 4. Effect of antimicrobial films on the growth of *E.coli* O157:H7 and *L. monocytogenes* on meat. Points represent average values (n = 3) and different letters for the data points at each sampling period indicate significant differences ($p < 0.05$).

Films containing SA had a decrease in *E. coli* O157: H7 and *L. monocytogenes* in 5 and 4 log CFU/g, while BA film had a decrease in 6 and 5 log CFU/g compared to control film at 5 °C during 12 days of storage. This reduction could be attributed to a decrease in the internal pH of microbial cells, produced by ionization of otherwise undissociated organic acid molecules, added to the disruption of substrate transport that leads to changes in the cell membrane permeability or reduction in its proton motive force, and also by chelation of metal ions that are essential for microbial growth (Ramos, Santos, et al., 2012). These results confirm that films produced by API incorporated with SA and BA were effective in controlling growth of *E. coli* O157: H7 and *L. monocytogenes*.

4. Conclusion

The physical and mechanical properties of protein isolate films were affected by the addition of different concentrations of SA or BA. The increase in concentrations of SA or BA resulted in greater thickness, color difference, opacity, elongation at break and water vapor permeability, but decreased tensile strength of the tested films. The micrographs of scanning electron microscopy revealed the surface micropores on films containing SA. The film containing BA had a rough surface, which facilitates the permeation of water vapor, resulting in an increase in water vapor permeability. The incorporation of different content of SA or BA in API films was the most effective in inhibiting the growth of *E. coli* O157:H7 and *L. monocytogenes*, while *S.* Enteritidis was the most resistant against lower content of organic acids incorporated films. *S. aureus* showed no inhibition at different levels and types of antimicrobial agents tested. The same inhibitory effect was observed when antimicrobial films were applied on meat against *E. coli* O157:H7 and *L. monocytogenes*. Films incorporated with SA had a decrease in 5 and 4 log CFU/g, while BA film had decrease of 6 and 5 log CFU/g of *E. coli* O157: H7 and *L. monocytogenes* compared to the control film. Therefore, an antimicrobial anchovy protein film incorporated with sorbic or benzoic acids is promising and seems to have good potential in food applications.

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