1 **Comparative study between film and coating packaging based on shrimp concentrate obtained from**
2 **marine industrial waste for fish sausage preservation**

3

4 A. Alemán, F. González, M.Y. Arancibia, M.E. López-Caballero, P. Montero and M.C. Gómez-Guillén

5 Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), CSIC (formerly Instituto del Frío), C/

6 José Antonio Nováis, 10, 28040. Madrid, Spain.

7 *Corresponding author: E-mail address: ailen@ictan.csic.es

8 Tel.: +34 91 5492300; fax: +34 91 5493627.

9

Abstract

An active shrimp concentrate (SC), obtained from *Litopenaeus vannamei* cooking juice, was incorporated into a chitosan-gelatin matrix to develop edible packaging covers (coating and film). Both types of packaging, which showed antimicrobial activity against some fish spoilers and pathogenic organisms, were applied to preserve fish sausages during chilled storage. The storage trial showed that the coating increased the lag phase of total viable microorganisms and enterobacteria to 15 and 10 days, respectively, while the film drastically inhibited growth of these groups. No presence of *Staphylococcus aureus* or lactic acid bacteria was detected in any batches during storage. Total volatile bases and pH values also decreased during storage, the decrease being more pronounced in the film. With regard to physical-chemical properties, the coated sausages exhibited values for moisture, water holding capacity, and shear strength similar to those of the control sausages until about day 25. The coating was imperceptible and could be a promising way of improving the quality of sausages during storage since it delayed microbial growth. The sausages wrapped in the film showed a drop in pH and moisture, as well as texture hardening, and were described as resembling a pickled product. The sausages were stable for at least 42 days, which represents a shelf life increase of at least this time with respect to the control sausages (in commercial plastic casings). The coating and the film had different effects on the physicochemical and microbiological properties of the fish sausage, and therefore on the stability of the product. In addition, the film triggered modifications in the product that resulted in a product different from the original, offering an attractive alternative with regard to product design.

Keywords: shrimp concentrate, chitosan-gelatin coating, film, antimicrobial, shelf life.

1. Introduction

Natural biopolymers, such as proteins, polysaccharides, lipids or mixtures of them, have been selected to develop biodegradable coatings and films. These coating and films can be edible and/or act as carriers of various active compounds (Tharanathan, 2003).

In this respect, it has previously been reported that a mixture of gelatin and chitosan can successfully extend the shelf life of foods such as patties, sausages and fillets of cod (López-Caballero et al., 2005a,b Gómez-Estaca et al., 2010). Chitosan, which has many excellent properties, is an antimicrobial polymer that inhibits the growth of bacteria, yeast and moulds. This activity is possible owing to the positive charges which can interact with negatively charged residues of macromolecules on the microbial cell surface, causing membrane leakage (Zheng & Zhu, 2003). On the other hand, gelatin, with its functional properties, contributes to the production of films and coatings with excellent physical and chemical properties (Gómez-Guillén et al., 2011).

Minced fish can act as base material for seafood product like fish sausages, which are gaining importance in recent years, because it is easy to digest and consumption of fish are good for human health. However, fish sausages are highly perishable because they are typically high in moisture and protein. Recently, a carotenoprotein concentrate from shrimp waste has shown promise of possessing active properties as an antimicrobial and antioxidant (Arancibia et al., 2014; Arancibia et al., 2015a). The incorporation of this concentrate into chitosan coatings extended the shelf life of shrimps (Arancibia et al., 2015b). In this regard, shrimp cooking juice (usually treated as waste) has also been studied as a valuable resource containing proteins, lipids and carotenoids with antioxidant and antimicrobial capacities (Pérez-Santín et al., 2013). However, to our knowledge, there is no information about the effect that the application of films and coatings based on gelatin-chitosan incorporating a carotenoid-protein concentrate obtained from cooking juice may have on storage of a fish product.

The aim of this work was to compare the effect of a formulation, in which the ingredients were obtained as waste from the seafood industry, both as a coating and as a film, on the shelf life and characteristics of a seafood product.

57

58 **2. Material and methods**

59 *2.1. Shrimp concentrate (SC)*

60 Shrimp (*Penaeus* spp.) cooking juice, supplied by Gambastar, S.L. (Burgos, Spain), was recovered
61 immediately before being sent to the water treatment plant and was frozen at –20 °C. The cooking juice
62 was thawed and passed through a centrifugal separator (GEA Westfalia Separator, OKA 2-06-566,
63 Düsseldorf, Germany) at 9560 rpm and constant flow rate (50 l/h), resulting in a pasty-looking
64 concentrate (SC) that was frozen at –80 °C until analysis. The shrimp concentrate showed approximately
65 68% moisture, 16% lipid, 10% protein and 1.4% ash.

66

67 *2.2. Coating and film preparation*

68 Chitosan (Guinama, Valencia, Spain, 85% deacetylation degree) was dissolved in 0.15 M lactic acid under
69 stirring overnight. Gelatin from warm-water fish (Lapi Gelatin, Florence, Italy) was dissolved in hot
70 distilled water (45 °C), and shrimp concentrate (SC) was suspended in distilled water containing Tween
71 80. Glycerol was used as plasticizer. All final solutions were homogenized with a T25 basic Ultra-Turrax
72 (IKA-Werke GmbH & Co. KG, D-79219 Staufen, Germany) at 17500–21500 rpm for 5 min and sonicated
73 (Q700, 132 Qsonica, CT, USA) at 70% amplitude.

74 The final solutions were: i) chitosan 1%, gelatin 1%, shrimp concentrate 2%, glycerol 0.2% and Tween
75 0.02%, w/v (SC-C batch) and ii) chitosan 1%, gelatin 1%, shrimp concentrate 1%, glycerol 0.2% and Tween
76 0.02%, w/v. 300 mL of the latter solution (containing 1% shrimp concentrate) was cast in dishes (35x23
77 cm) and dried in an oven (FD 240, Binder, Tuttlingen, Germany) at 45 °C for 21 h. The dried film (SC-F
78 batch) was conditioned at 58% RH and 22 °C for 4 days prior to analysis. An attempt was made to
79 develop a film with 2% of the concentrate, but it was not possible; the resulting film was too thick and
80 brittle and could not be removed from the plate.

81

82 *2.3. Antimicrobial activity in vitro*

The antimicrobial activity of the shrimp concentrate, coating and edible film (SC, SC-C and SC-F, respectively) was determined by the disk diffusion method in agar as previously described by Giménez et al. (2012). Paper disks 5 mm in diameter soaked with 40 µl of the solutions (shrimp concentrate or coating), and 5 mm disks of the edible film (obtained directly) were laid on the surface of agar previously inoculated with microorganisms ($\approx 10^5$ cfu/mL). The strains, selected for their importance for health (such as probiotics or pathogens) or for being responsible for fish spoilage, were obtained from the Spanish Type Culture Collection (CECT): *Aeromonas hydrophila* CECT 839T, *Aspergillus niger* CECT 2088, *Bacillus cereus* CECT 148, *Bacillus coagulans* CECT 56, *Bifidobacterium animalis* subspecies *lactis* DSMZ 10140, *Bifidobacterium bifidum* DSMZ 20215, *Brochothrix thermosphacta* CECT 847, *Citrobacter freundii* CECT 401, *Clostridium perfringens* CECT 486, *Debaryomyces hansenii* CECT 11364, *Enterococcus faecium* DSM 20477, *Escherichia coli* CECT 515, *Lactobacillus acidophilus* CECT 903, *Lactobacillus helveticus* DSM 20075, *Listeria innocua* CECT 910, *Listeria monocytogenes* CECT 4032, *Penicillium expansum* DSMZ 62841, *Photobacterium phosphoreum* CECT 4192, *Pseudomonas aeruginosa* CECT 110, *Pseudomonas fluorescens* CECT 4898, *Salmonella Choleraesuis* CECT 4300, *Shewanella putrefaciens* CECT 5346T, *Shigella sonnei* CECT 4887, *Staphylococcus aureus* CECT 24, *Vibrio parahaemolyticus* CECT 511T and *Yersinia enterocolitica* CECT 4315. Strains were grown in BHI broth (Oxoid) (supplemented with 3% NaCl for *V. parahaemolyticus* and 1% NaCl for *P. phosphoreum*) for 24 h. The microorganisms were incubated at 37 °C (with the exception of *A. hydrophila*, *P. fluorescens* and *S. putrefaciens*, which were incubated at 30 °C, *B. thermosphacta* at 25 °C and *P. phosphoreum* at 15 °C). In addition, *L. acidophilus* was incubated in an incubator with 5% CO₂ and *C. perfringens* under anaerobic conditions (Gas-Pack, Anaerogen, Oxoid). After incubation, the inhibition diameter (considered to be the antimicrobial activity) was measured with the Corel Photo-Paint X3 software. Results were expressed as diameter of growth inhibition (mm). Each determination was performed twice in duplicate.

2.4. Fish storage trial

2.4.1. Sample preparation

Frozen surimi blocks of Alaska pollock (*Theragra chalcogramma*), stored frozen at –20 °C, were supplied by Congalsa (A Coruña, Spain). The ingredients added to prepare the different samples were NaCl, starch and egg white from Panreac AppliChem (Barcelona, Spain).

Prior to use (24 h), surimi was taken from the –20 °C store and thawed in a 4 °C refrigerator for easy cutting into smaller pieces. Then the surimi was chopped and homogenized for 1500 rpm/10 min at –2 °C in a vacuum homogenizer (FD112M10-72D, Stephan Machinery GmbH, Hameln, Germany), and some ingredients were added as follows: water (as ice) to obtain a final moisture of 76%, NaCl (2%), starch (5%) and egg white (1%). The mixture was homogenized to form a bright, homogeneous, viscous mass.

For the preparation of the sausages, the resulting dough was manually stuffed into 20 mm NOJAX cellulose casings (Viscase SA, Bagnold Cedex, France). Sausages were heated in an Eller oven (Unimatic 1000, Micro 40, Eller, Merano) at 90 °C for 30 min. Once the heating treatment was finished, the sausages were dipped into ice water to cool them quickly. Before further treatment, the cellulose casing was removed from the sausages.

The sausages obtained (approximately 150 g each) were divided into three different batches according to the packaging method. A group of sausages wrapped in commercial transparent plastic film was used for control purposes (batch S); other sausages were wrapped with film made from chitosan, gelatin and 1% shrimp concentrate (batch S-SC-F), and finally the remaining group was dipped into a solution of chitosan, gelatin and 2% shrimp concentrate for 2 minutes and allowed to drain for 1 min (batch S-SC-C). All samples were stored in a cooling chamber at 5 °C. The experiment was performed twice.

2.4.2. Microbiological assays

The microbiological analyses were as follows: 10 g of sausages (from at least 3 different packages), were mixed with 90 ml of buffered 0.1% peptone water (Oxoid, Basingstoke, UK) using a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) in a Stomacher blender (model Colworth 400, Seward, London, UK) during 1 min. Appropriate dilutions were prepared for the following microorganism determinations: total bacterial counts (TBC) on pour plates of Plate Count Agar (PCA) incubated at 30 °C for 72 h;

Enterobacteriaceae on double-layered plates of Violet Red Bile Glucose agar (VRBG, Oxoid) incubated at 30 °C for 48 h; lactic acid bacteria on double-layered plates of De Man, Rogosa and Sharpe (MRS) agar (Oxoid) incubated at 30 °C for 72 h.; *Staphylococcus aureus* coagulase positive on Baird Parker agar (BioMérieux S.A., Marcy l'Etoile, France) supplemented with lyophilized rabbit plasma (bioMérieux). Microbiological counts are expressed as the log of the colony-forming units per gram (log cfu/g) of sample. All analyses were performed in triplicate in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain).

2.4.3. pH

The sausages were homogenized (1:10 w/v) with distilled water using the T25 basic Ultra-Turrax. The pH was determined with a pHm93 pH-meter and a combined pH electrode (Radiometer, Copenhagen, Denmark). The experiments were performed at least in triplicate.

2.4.4. Total volatile basic nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) determinations were carried out according to the method of Antonacopoulos & Vyncke (1989). 10 g $\pm$ 0.1 g of the sausage was homogenized with 90 ml 6% perchloric acid solution for two minutes. The mixture obtained was filtered through Whatman No. 1 paper, washed with 5 mL of perchloric acid, and adjusted to 100 mL. After filtering, the extract was alkalinized with 20% sodium hydroxide solution and submitted to steam distillation in a Tecator AB device (model 1002, Kjeltex Systems, Sweden). The distillate was collected on boric acid (0.3% w/v) and was titrated with 0.05 M HCl. At least three replications were performed, and the results were expressed as mg TVB-N/100 g sample.

2.4.5. Water content

Water content was determined in accordance with the AOAC method (2005).

2.4.6. Water holding capacity (WHC)

WHC was determined as previously described by Sánchez-Alonso, Haji-Maleki & Borderías (2006) with some modifications. 1.5 g of sausage was placed in tubes with absorbent paper (previously dried at 105 °C) and centrifuging at $4000 \times g$ at 20 °C for 15 min in a Sorvall model RT6000B centrifuge (Dupont Co., Wilmington, DE, USA). WHC was expressed as the amount of retained water per 100 g of water present in the sample before centrifuging. Analyses were performed at least in triplicate.

2.4.7. Colour parameters

The colour parameters were measured using a Konica Minolta CM-3500d colorimeter (Osaka, Japan). Measurements were taken at different locations in the sausages and each point is the mean of at least nine measurements. Colour was expressed as L^* , a^* , and b^* , indicating luminosity, chromaticity on a green (–) to red (+) axis, and chromaticity on a blue (–) to yellow (+) axis, respectively (Chen et al., 2010).

2.4.8. Shear strength

Sausage hardness was measured as the shear strength of samples using a Kramer shear cell (HDP/MK05). Measurements were taken at a cross-head speed of 100 mm/min using a 5-kN load cell in an ANAME texturometer (TA-XT Plus, NY, USA). Readings were taken using the maximum force setting and have been expressed as the sample to weight ratio (N/g). The values given are the mean of five replications.

2.4.9. Sensory evaluation

A sensory analysis of the sausages was performed on the 15th day of storage. A panel of fifteen semi-trained judges was used to evaluate overall acceptability on a 5-point scale: 1, dislike very much, and 5, like very much. The judges were also asked to describe the samples. Sausages were sliced into 2 cm thick pieces and served to each panellist separately under white illumination. Water was provided for cleansing the palate between samples.

2.5. Statistical analyses

Results were expressed as mean $\pm$ standard deviation. Results were analysed by two-way ANOVA (two factors: batch and storage time). Means were tested with Tukey's test for paired comparison, with a significance level of $\alpha = 0.05$, using the SPSS computer programme (SPSS Statistical Software Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Antimicrobial activity

The antimicrobial activity of the shrimp concentrate (SC) and the gelatin-chitosan coating solution and film (SC-C and SC-F, respectively) are shown in Table 1. The shrimp concentrate inhibited microbial growth of Gram-positive and Gram-negative bacteria, including fish pathogens (e.g. *Y. enterocolitica*) and organisms involved in fish spoilage (e.g. *S. putrefaciens*, *P. phosphoreum*). During recovery of this shrimp concentrate, it might be possible to obtain a peptide fraction active against microorganisms. For example, Rosa & Barracco (2010) found antimicrobial peptides in shrimp (*Penaeus* sp.), while Arancibia et al. (2014) obtained a protein-lipid concentrate with antimicrobial activity from shrimp waste. The latter authors reported a significant amount of hydrophobic amino acid residues in the composition of the concentrate, as well as arginine, proline, glycine, tryptophan and other amino acids. Since the species used in the present work is the same as the one used by those authors, residues with a similar composition might be responsible for the SC antimicrobial activity.

The activity of the SC remained when it was incorporated into the chitosan-gelatin coating solution, or even increased, as in the case of *A. hydrophila*, *E. coli* and *S. Choleraesuis* (Table 1). The antimicrobial effect of chitosan is well known, and, although its activity depends on several factors such as molecular weight, degree of deacetylation, conformational structure, etc. (Rabea et al., 2003), one possibility regards its activity as a change in cell permeability due to interactions between chitosan (polycationic polymer) and cell surfaces (negative charges). This leads to the loss of intracellular electrolytes and proteinaceous constituents (Jung et al., 1999). Similarly, protein residues from concentrate might be

charged positively and thus exerts their activity or increase the activity of chitosan. On the other hand, Devlieghere et al. (2004) reported that chitosan was generally more effective against Gram-positive than Gram-negative bacteria. In the present work, the microorganisms most sensitive to the coating solution were mainly Gram-negative. A protein-lipid concentrate isolated from shrimp waste increased the antimicrobial activity of a chitosan coating (Arancibia et al., 2014). The formulations that were tested did not affect the growth of potentially probiotic microorganisms (*L. acidophilus* and *Bifidobacterium*) (Table 1). The film inhibited the growth of most of the microorganisms studied, but its activity is limited to the agar in contact with the film. The solid state and the lower water concentration in the film might favour sorption by free active groups, thus restricting antimicrobial activity to a specific area (Elsabee & Abdou, 2013; Arancibia et al., 2014).

3.2. Fish storage trial

3.2.1. Microbiological analyses

Since the formulations showed antimicrobial activity against organisms involved in fish spoilage or potentially pathogenic microorganisms, we planned to study the role of these coatings and films during chilled storage of Alaska pollock sausages. Initially, as expected, total microbial counts were below the detection limit in all the batches owing to the effect of heat treatment. In control sausage (S), total mesophilic microorganisms increased exponentially in early stages of preservation, reaching 9 log cfu/g at 22 days (Fig. 1a). These counts are higher than the 10^6 cfu/g obtained in sardine sausages stored at 2 ± 1 °C (Ravishankar et al., 1992) for 26 days. Raju et al. (2003) reported counts of the order of 10^7 cfu/g in refrigerated sausages made from Japanese threadfin bream (*Nemipterus japonicus*) at 30 days.

The application of active packaging, both as coating and as film, increased the lag phase of microorganisms to 15 days, with an inhibition higher than 7 log cycles compared with the control batch (Fig. 1a). At that point, exponential growth started in the coating batch (S-SC-C), reaching 8 log cfu/g at 42 days. Sausage wrapped in film (S-SC-F) remained below the detection limit throughout the storage period studied. Soares et al. (2013) found that chitosan coatings (0.25, 0.50 and 0.75% w/v) can be a

good barrier to protect frozen fish from deterioration after defrosting, since total viable counts and total volatile basic nitrogen were maintained below the maximum limits recommended (5×10^5 cfu/g and 35 mg nitrogen/100 g fish, respectively).

Enterobacteria evolved in a similar manner to the mesophilic microorganisms (Fig. 1b). The control batch (S) exceeded 3 log cfu/g at 8 days, while S-SC-C reached that level at 15 days ($p \leq 0.05$). After that S-SC-C registered lower values (approx. 4 log units) than S at 22 days. The incorporation of chitosan (1.5%) solution into formulas of pressurized cod sausages prevented the growth of enterobacteria for up to 20 days during storage at 2 ± 1 °C (López-Caballero et al., 2005b). In the present work, enterobacteria counts in S-SC-F sausage remained below the detection limit during storage (Fig. 1b).

Lactic acid bacteria were not detected; therefore possible changes in the product due to the action of the lactic group (Leroi, 2010) were dismissed. Contamination by *S. aureus* was also assessed. The presence of this organism implies poor quality of raw materials, improper handling of samples or post-process contamination. However, in this study the presence of *S. aureus* was not detected in any of the batches.

Microbiological analyses of the sausages with active coating and films revealed a drastic decrease or even inhibition of microbial growth (Figs. 1a, b). This was particularly pronounced in the S-SC-F batch ($p \leq 0.05$). Some authors report a gradual increase in the microbial load in various foods throughout preservation, which is reduced with the application of a gelatin-chitosan coating on fish patties (López-Caballero et al., 2005b), fruit and vegetables (Soares, 2009) and shrimps (Arancibia et al., 2015b).

The incorporation of a carotenoprotein concentrate into a chitosan coating increased the antimicrobial activity of the coating (Arancibia et al., 2014; Arancibia et al., 2015b). Those authors stated that this might be because the concentrate preparation involves some hydrolysis processes which could allow some antimicrobial peptide fractions to exert activity. Moreover, at the (acid) pH at which the chitosan is dissolved, the positively charged C-2 of the glucosamine monomer causes the chitosan chain to be further extended (Huang et al., 2004), thus enhancing solubility and antimicrobial activity. Furthermore, the acid pH favours the chitosan and the proteins being positively charged (Devlieghere et al., 2004). As

mentioned earlier, both chitosan and proteins could interact with the negative charge remaining on the surface of the bacteria, which would compromise the integrity of the membrane and thus improve the antimicrobial effect. In the present work, the pH of the coatings and films (≈ 6.2) was increased by incorporating the protein concentrate at basic values (≈ 6.5), but the antimicrobial activity was maintained in both cases.

3.2.2. pH

Initially, the pH of the sausages was close to neutral (6.83), with a small decrease in the S batch, reaching 6.57 at 22 days ($p \leq 0.05$). The pH values in the present work were similar to those found in chilled cooked Japanese Baga sausages (*N. japonicus*) at 30 days (Raju et al., 2003). The pH was maintained around 6.26 ($p \leq 0.05$) in S-SC-C until the end of the storage period. However, the pH dropped sharply in the S-SC-F batch, with values lower than 5 ($p \leq 0.05$) at 42 days. This was attributed to the film, which could capture some free water from the sausage and thus facilitate the passage of the lactic acid in which the chitosan was dissolved to the gel (Fig. 2a), and consequently reduce microbial growth by limiting the effect of pH (Figs. 1a, b).

3.2.3. Total volatile basic nitrogen (TVB-N)

TVB-N was 1.12 mg/100 g sample in newly cooked sausage. During storage, the control sausage (S) reached 5.14 mg TVB-N/100 g at 22 days, while the other batches remained below even after 42 days of storage (Fig. 2b). López-Caballero et al. (2005b) obtained values lower than 10 mg/100 g TVB-N in cod sausages formulated with chitosan solution. In the present work, levels of volatile bases were not related to the microbial counts. As the sausages were cooked, the thermal treatment modified the microbiota and hence the spoilage pattern. The major microbiota developed in this product could then produce lower levels of basic compounds. The production of TVB-N in the present work was well below the limits set for fresh fish by the European Union (30 mg TVB-N/100 g muscle, EEC, 1995). The low production of volatile compounds was consistent with the pH values registered (Figs. 2a, b).

3.2.4. Water content

S-SC-F recorded around 6% lower water than the S and S-SC-C batches ($p \leq 0.05$), which is attributed to absorption of water by the film (Fig. 3a). At the end of storage, S-SC-F showed water content values of $68.33 \pm 0.08\%$ (Fig. 3a), which was similar to those obtained in Alaska pollock surimi gel nutrified with ω -3 polyunsaturated fatty acids (Pietrowski, 2011). Dincer & Calkri (2010) reported lower water content (52.73%) in rainbow trout sausages, with WHC values of about 97%.

3.2.5. Water holding capacity (WHC)

The WHC, which indicates how the water is retained in the cooked product, was homogeneous in the three batches, being about 95% during storage (Fig. 3b) ($p \leq 0.05$). These results are similar to those reported for Alaska pollock surimi products with wheat fibre (Sánchez-Alonso, Haji-Maleki & Borderías, 2006).

3.2.6. Colour

Colour parameters, L^* (lightness), a^* (redness) and b^* (yellowness), of sausage during storage are shown in Figure 4. The L^* , a^* and b^* values of the freshly cooked sausage were 75.23 ± 0.33 , -4.04 ± 0.05 and 0.80 ± 0.11 , respectively. In the control sausage, L^* and a^* remained practically constant during storage, but a slight decrease in yellowness was observed up to day 15. The lightness was similar to that obtained in sausages of threadfin bream surimi (*N. japonicus*) (Santana et al., 2015) and Alaska pollock surimi (Sell et al., 2015), the same species as the one used in this experiment. However, the latter authors reported values of a^* (≈ 9.55) and b^* (≈ 36.25) that differ greatly from those obtained in the present work and those observed by Pietrowski et al. (2011) ($L=82.20$, $a=-3.41$, $b=3.72$) for an Alaska pollock surimi gel. This could be attributed to the different ingredients used in the preparation of the sausages.

The application of a coating or film during storage barely altered the lightness and a^* values ($p \leq 0.05$) of the sausages (Fig. 4). Arancibia et al. (2015b) indicated that during shrimp storage the incorporation of a

protein concentrate from shrimp waste in the chitosan coating contributed slightly to the maintenance of lightness (L^*), while showing a marked tendency towards red (a^*). With regard to the tendency to yellow or blue (b^*), it was observed that in S-SC-C this parameter decreased slightly (3 points) between days 0 and 15, and then remained constant. In S-SC-F, however, after decreasing until day 15, b^* values then increased significantly (more than ten times). Changes in b^* values were more pronounced in S-SC-F than in the S-SC-C batch ($p \leq 0.05$). Although both contained chitosan (slightly yellowish colour) and concentrate (orange-yellow colour) in their composition, the film (which was very soluble) permanently wrapped the sausage and therefore the sausage could be coloured more easily. In contrast, the coating formed a very fine layer that could drain away after application, giving less colouring to the sausage (Fig. 4). Moreover, water content loss in S-SC-F could also contribute to a greater tendency to yellow, owing to the higher concentration of the sausage ingredients. Reppond & Babbitt (1997) found that b^* values for pollock surimi gel decreased linearly with increased moisture content.

3.2.7. Shear strength

Figure 5 plots the Kramer test results. With initial values around 25 N/g, S and S-SC-C remained practically constant during storage, except for a slight increase in the S-SC-C batch at 42 days. However, S-SC-F evolved very differently, with the values increasing to 120 N/g ($p \leq 0.05$) in the final stages, which gives an indication of higher resistance to compression force. The surface dehydration in this batch, caused by absorption of water by the film, represented a thickness reduction of 12% in the S-SC-F sausage, which produced a compacted structure resulting in a significant increase in hardness. The Kramer force for S-SC-F was similar to those reported for various Alaska pollock surimi gels prepared with different formulations (Pietrowski et al., 2011). It has previously been reported that a chitosan coating, with or without the incorporation of polyethyleneimine or thyme, prevents softening and loss of texture during cold preservation of snakehead (*Channa argus*) (Yang et al., 2015). López-Caballero et al. (2005a) reported that the hardness of cod patties coated with gelatin-chitosan was higher than that of uncoated ones during chilled storage. García et al. (2008) reported that a chitosan coating (0.5 to 1% w/v)

increased the hardness of cooked ham compared with control ham during cold preservation. Hardness and some other texture parameters were higher for chitosan-coated sardine than for untreated samples, and they increased with the storage period and were well correlated with WHC and drip loss (Mohan et al., 2012).

3.2.8. Sensory analysis

Given the different textures found in the first two weeks, a sensory analysis of the sausages was performed to determine the overall acceptability for consumers. The 15th day of storage was selected for the analysis because S and S-SC-C were microbiologically acceptable and the pH of S-SC-F remained constant. Although the products were obviously different, the panellists favourably approved all the fish sausages, with mean scores of 4.02 ± 0.53 , 3.82 ± 0.37 and 3.68 ± 0.43 for S, S-SC-C and S-SC-F, respectively, on a 5-point scale. The sausage wrapped in film was described by 60% of the panellists as resembling a slightly pickled product.

Conclusions

The application of an antimicrobial coating of chitosan-gelatin enriched with a shrimp concentrate was imperceptible, and helped to extend the shelf life of a fish sausage to 15 days. With the application of the corresponding film, the resulting sausages exhibited a harder texture and lower pH and water content, resembling a firm, slightly pickled product, which provided greater microbiological control. The application of a film to a fish sausage could be an option for the design of products resembling pickled products which are during storage, and this could be the subject of further studies.

Acknowledgements

This research was financed by the Spanish Ministry of Economy and Competitiveness through projects AGL2011-27607, 201370E036, and AGL2014-528225. Author M. Arancibia was funded by a SENESCYT scholarship provided by the Ecuadorian government.

369 ** This centre has implemented and maintains a Quality Management System which fulfils the
370 requirements of the ISO standard 9001:2000.
371

372 **References**

- 373 Antonacopoulos, N., & Vyncke, W. (1989). Determination of volatile basic nitrogen in fish: A third
374 collaborative study by the West European Fish Technologists' Association (WEFTA). *Zeitschrift für*
375 *Lebensmittel-Untersuchung und Forschung*, 189(4), 309–316.
- 376 AOAC (2005). Official Methods of Analysis. (18th ed). Gaithersburg, MD, USA: Association of Official
377 Analytical Chemists.
- 378 Arancibia, M. Y., Alemán, A., Calvo, M., López-Caballero, M. E., Montero, P., & Gómez-Guillén, M. C.
379 (2014). Antimicrobial and antioxidant chitosan coatings enriched with active shrimp (*Litopenaeus*
380 *vannamei*) waste materials. *Food Hydrocolloids*, 35, 710–717.
- 381 Arancibia, M. Y., López-Caballero, M. E., Gómez-Guillén, M. C., & Montero, P. (2015b). Chitosan coatings
382 enriched with active shrimp waste for shrimp preservation. *Food Control*, 54, 259–266.
- 383 Arancibia, M., Alemán, A., López-Caballero, M. E., Gómez-Guillén, M. C. and Montero, P. (2015a).
384 Development of active films of chitosan isolated by mild extraction with added protein concentrates
385 from shrimp waste. *Food Hydrocolloids*, 43, 91–99.
- 386 Benjakul, S., Visessanguan, W., Tanaka, M., Ishizaki, S., Suthidham, R., & Sungpech, O. (2001). Effect of
387 chitin and chitosan on gelling properties of surimi from barred garfish (*Hemiramphus far*). *Journal of the*
388 *Science of Food and Agriculture*, 81(1), 102–108.
- 389 Chen, Z., Zhu, C., Zhang, Y., Niu, D., & Du, J. (2010). Effects of aqueous chlorine dioxide treatment on
390 enzymatic browning and shelf-life of fresh-cut asparagus lettuce (*Lactuca sativa* L.). *Postharvest Biology*
391 *and Technology*, 58(3), 232–238.
- 392 Coma, V., Martial-Gros, A., Garreau, S., Copinet, A., Salin, F., & Deschamps, A. (2002). Edible
393 antimicrobial films based on chitosan matrix. *Journal of Food Science*, 67(3), 1162–1169.
- 394 Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J., & Bachère, E. (1997). Penaeidins, a
395 new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *Journal of*
396 *Biological Chemistry*, 272(45), 28398–28406.

397 Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: antimicrobial activity, interactions with
 398 food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, 21, 703–714.
 399 Dincer, T., & Cakli, S. (2010). Textural and Sensory Properties of Fish Sausage from Rainbow Trout.
 400 *Journal of Aquatic Food Product Technology*, 19, 238–248.
 401 EC. The Commission of the European Communities 95/149/EC (1995). Determination of the
 402 concentration of volatile nitrogenous bases (TVB-N) in fish and fish products: a reference procedure.
 403 *Official Journal of the European Communities*, 97, 84–87.
 404 Elsabee, M. Z, & Abdou, E. S. (2013). Chitosan based edible films and coatings: a review. *Material Science*
 405 *and Engineering*, 33(4), 1819-1841.
 406 Garcia, M. A., Diaz, R., Gate, H., Beldarraín, T., Castillo, A., González, J., & Duarte, C. (2008). Active
 407 packaging of pork sausages. *Food Science and Technology*, 18(2), 1–7.
 408 Giménez, B., Gómez-Guillén, M. C, López-Caballero, M. E., Gómez-Estaca, J., & Montero, P. (2012). Role
 409 of sepiolite in the release of active compounds from gelatin-egg white films. *Food Hydrocolloids*, 27(2),
 410 475-486.
 411 Gómez-Estaca, J., López de Lacey, A., López-Caballero, M. E., Gómez-Guillén, M. C., & Montero, P. (2010).
 412 Biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish
 413 preservation. *Food Microbiology*, 27, 889–896.
 414 Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E., & Montero, M. P. (2011). Functional and
 415 bioactive properties of collagen gelatin from alternative sources: A review. *Food Hydrocolloids*, 25, 1813–
 416 1827.
 417 Huang, R., Du, Y., Zheng, L., Liu, H., & Fan L. (2004). A new approach to chemically modified chitosan
 418 sulfates and study of their influences on the inhibition of Escherichia coli and Staphylococcus aureus
 419 growth. *Reactive and Functional Polymers*, 59(1), 41–51.
 420 Jeon, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by
 421 bioreactor. *Carbohydrate Polymers*, 44(1), 71–76.

422 Jung, B., Kim, C., Choi, K., Lee, Y. M., & Kim, J. J. (1999). Preparation of amphiphilic chitosan and their
 423 antimicrobial activities. *Journal of Applied Polymer Science*, 72(13), 1713–1719.

424 Kołodziejska, I., Piotrowska, B., Bulge, M., & Tylingo, R. (2006). Effect of transglutaminase and 1-ethyl-3-
 425 (3-dimethylaminopropyl) carbodiimide on the solubility of fish gelatin-chitosan films. *Carbohydrate*
 426 *Polymers*, 65(4), 404–409.

427 Leroi, F. (2010). Occurrence and role of lactic acid bacteria in seafood products. *Food Microbiology*, 27
 428 (6), 698–709.

429 López-Caballero, M. E., Gómez-Guillén, M. C., Pérez-Mateos, M., & Montero, P. (2005a). A chitosan-
 430 gelatin blend as a coating for fish patties. *Food Hydrocolloids*, 19, 303–311.

431 López-Caballero, M. E., Gómez-Guillén, M. C., Pérez-Mateos, M., & Montero, P. (2005b). A functional
 432 chitosan-enriched fish sausage induced by high pressure. *Journal of Food Science*, 70(3), 166–71.

433 Mohan, C. O., Ravishankar, C. N., Lalitha, K. V., & Srinivasa, T. K. (2012). Effect of chitosan edible coating
 434 on the quality of double filleted Indian oil sardine (*Sardinella longiceps*) during chilled storage. *Food*
 435 *Hydrocolloids*, 26(1), 167–174.

436 Pérez-Santín, E., Calvo, M. M., López-Caballero, M. E., Montero, P., & Gómez-Guillén, M. C. (2013).
 437 Compositional properties and bioactive potential of waste material from shrimp cooking juice. *LWT -*
 438 *Food Science and Technology*, 54, 87–94.

439 Pietrowski, B. N., Tahergorabi, R., Matak, K. E., Tou, J. C., & Jaczynski, J. (2011). Chemical properties of
 440 surimi seafood nutrified with ω -3 rich oils. *Food Chemistry*, 129(3), 912–919.

441 Rabea, E. I., Badawy, M. E., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as
 442 Antimicrobial Agent: Applications and Mode of Action. *BioMacromolecules*, 4(6), 1457–1465.

443 Raju, C. V., Shamasundar, B. A., & Udupa, K. S. (2003). The use of nisin as a preservative in fish sausage
 444 stored at ambient (28 ± 2 °C) and refrigerated (6 ± 2 °C) temperatures. *International Journal of Food*
 445 *Science & Technology*, 38, 171–185.

446 Ravishankar, C. N., Setty, T. M. R., & Shetty, T. S. (1992). Method for the preparation of sausages of
 447 acceptable quality from Indian oil sardine (*Sardinella longiceps*) and their shelf-life at different storage
 448 temperatures. *Food Control*, 3, 144–148.

449 Rosa, R., & Barracco, M. (2010). Antimicrobial peptides in crustaceans. *Invertebrate Survival Journal*, 7,
 450 262–284.

451 Sánchez-Alonso, I., Haji-Maleki, R., & Borderías, A. J. (2006). Effect of wheat fibre in frozen stored fish
 452 muscular gels. *European Food Research and Technology*, 223(4), 571–576.

453 Santana, P., Huda, N., & Yang, T. A. (2015) Physicochemical properties and sensory characteristics of
 454 sausage formulated with surimi powder. *Journal of Food Science and Technology*, 52(3), 1507–15015.

455 Sell, C., Beamer, S., Jaczynski, J., & Matak, K. E. (2015). Sensory characteristics and storage quality
 456 indicators of surimi franks nutritionally enhanced with omega-3 rich flaxseed oil and salmon oil.
 457 *International Journal of Food Science and Technology*, 50, 210–217

458 Soares, N. F. (2009). Chitosan-Properties and Application (chapter 5). In Long Yu (Ed.), *Biodegradable*
 459 *Polymer Blends and Composites from Renewable Resources* (pp. 107–127). J. Wiley & Sons, Inc.

460 Soares, N. M., Tânia, T. S, Mendes, S., & Vicente, A. A. (2013). Effect of chitosan-based solutions applied
 461 as edible coatings and water glazing on frozen salmon preservation – A pilot-scale study. *Journal of Food*
 462 *Engineering*, 116, 316–323.

463 Tharanathan, R. (2003). Biodegradable films and composite coatings: Past, present and future. *Trends in*
 464 *Food Science & Technology*, 14(3), 71–78.

465 Váscónez, M. B., Flores, S. K., Campos, C. A., Alvarado, J., & Gerschenson, L. N. (2009). Antimicrobial
 466 activity and physical properties of chitosan-tapioca starch based edible films and coatings. *Food Research*
 467 *International*, 42(7), 762-769.

468 Yang, F., Hu, S., Lu, Y., Yang, H., ZHAO, Y., & LI, L. (2015). Effects of Coatings of Polyethyleneimine and
 469 Thyme Essential Oil Combined with Chitosan on Sliced Fresh *Channa argus* during Refrigerated Storage.
 470 *Journal of Food Process Engineering*, 38, 225–233.

471 Zheng, L. Y & Zhu, J. F. (2003). Study on antimicrobial activity of chitosan with different molecular
472 weights. *Carbohydrate Polymers* 54, 527–530.
473

Table 1. Antimicrobial activity (cm of inhibition growth).

Microorganism	SC	SC-C	SC-F
<i>Aeromonas hydrophila</i>	0.66±0.03 ^b	0.80±0.01 ^c	0.50±0.00 ^a
<i>Aspergillus niger</i>	-	-	-
<i>Bacillus cereus</i>	-	-	0.50±0.00
<i>Bacillus coagulans</i>	0.68±0.02 ^b	0.73±0.09 ^b	0.50±0.00 ^a
<i>Bifidobacterium animalis</i>	-	0.75±0.05 ^b	0.50±0.00 ^a
<i>Bifidobacterium bifidum</i>	-	0.66±0.00 ^b	0.50±0.00 ^a
<i>Brochothrix thermosphacta</i>	0.72±0.05 ^b	0.79±0.02 ^b	0.50±0.00 ^a
<i>Citrobacter freundii</i>	0.56±0.01 ^a	0.63±0.05 ^b	0.50±0.00 ^a
<i>Clostridium perfringens</i>	-	0.69±0.01 ^b	0.50±0.00 ^a
<i>Debaryomyces hansenii</i>	-	-	-
<i>Enterococcus faecium</i>	-	-	0.50±0.00
<i>Escherichia coli</i>	0.64±0.02 ^b	0.73±0.01 ^c	0.50±0.00 ^a
<i>Lactobacillus acidophilus</i>	-	-	-
<i>Lactobacillus helveticus</i>	-	-	-
<i>Listeria innocua</i>	0.64±0.04 ^b	0.66±0.00 ^b	0.50±0.00 ^a
<i>Listeria monocytogenes</i>	0.58±0.01 ^b	0.58±0.02 ^b	0.50±0.00 ^a
<i>Penicillium expansum</i>	-	-	-
<i>Photobacterium phosphoreum</i>	0.76±0.01 ^b	0.75±0.01 ^b	0.50±0.00 ^a
<i>Pseudomonas aeruginosa</i>	-	0.57±0.00 ^b	0.50±0.00 ^a
<i>Pseudomonas fluorescens</i>	0.57±0.00 ^b	0.58±0.03 ^b	0.50±0.00 ^a
<i>Salmonella Choleraesuis</i>	0.69±0.02 ^b	0.75±0.02 ^c	0.50±0.00 ^a
<i>Shewanella putrefaciens</i>	0.57±0.05 ^a	0.53±0.01 ^a	0.50±0.00 ^a
<i>Shigella sonnei</i>	0.66±0.01 ^b	0.67±0.00 ^b	0.50±0.00 ^a
<i>Staphylococcus aureus</i>	0.57±0.02 ^b	0.68±0.01 ^c	0.50±0.00 ^a
<i>Vibrio parahaemolyticus</i>	-	-	-
<i>Yersinia enterocolitica</i>	1.23±0.03 ^c	0.86±0.03 ^b	0.50±0.00 ^a

SC: shrimp concentrate; SC-C: Coating of chitosan-gelatin enriched with shrimp concentrate;
 SC-F: Film of chitosan-gelatin enriched with shrimp concentrate."-": without inhibitory activity

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5