Effects of agar films incorporated with fish protein hydrolysate or clove essential oil on flounder (*Paralichthys orbignyanus*) fillets shelf-life

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Abstract

This study evaluated the effect of protein hydrolysate (PH) and clove essential oil (CEO) on agar film properties and the shelf-life improvement of flounder fillets. Firstly, Argentine croaker muscle protein was alkali-solubilised and recovered to obtain a stable substrate. This substrate was hydrolysed to 20% degree of hydrolysis, by Alcalase (A20) and Protamex (P20). The molecular weight (MW) distribution, amino acid and in vitro antimicrobial activity of these hydrolysates were tested. Agar films were prepared with and without (control) incorporation of PH (A20 hydrolysate) or CEO characterised by mechanical properties, water vapour permeability, solubility and optical properties. The microbiological, physical and chemical properties of flounder fillets coated with these films were monitored during storage at 5°C for 15 days. Compared to P20, A20 hydrolysate possessed lower MW, higher hydrophobic amino acid content and the most effective antimicrobial activity against selected pathogens, such as Staphylococcus aureus, Yersinia enterocolitica, Aeromonas hydrophila, Debaryomyces Hansenii and Listeria innocua. The presence of PH increased the water solubility, water vapour permeability, elongation at break and yellowness of the films. The CEO films possessed less transparency than PH films and control films, due to the presence of oil in the formulations. Fillets covered with PH and CEO films, especially the latter, presented lower total volatile bases and pH values, and delayed considerably the growth of H₂S-producing microorganisms compared to the control films. The CEO might be used as a natural biopreservative to extend the flounder shelf-life. However, further studies are necessary, to assess the antimicrobial effect of PH when used as food packaging.

Keywords: Argentine croaker, Film, Agar, Clove essential oil, Fish protein hydrolysate, Natural preservative
The microbial growth from contamination or natural sources, during refrigerated storage, leads to fish spoilage (Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010; López de Lacey, López-Caballero, & Montero, 2014). The flounder (Paralichthys orbignyanus) is distributed from Rio de Janeiro (Brazil) to Patagonia (Argentina), in the southwest of the Atlantic, constituting a valuable fishing resource in these countries (Bolasina, 2011; Millner, Walsh, Díaz, & Astarloa, 2005). The microbial spoilage of fish can manifest itself as visible growth (slime), textural changes, and generation of off-odours and off-flavours (Gram & Huss, 2010; Van Haute, Raes, Devlieghere, & Sampers, 2017). The main microorganisms responsible for the degradation of fish are rod-shaped Gram-negative bacteria, belonging to the genera Pseudomonas, Moraxella, Vibrionaceae and Shewanella, among others (Gram & Huss, 2010). However, the stress created by additional antimicrobial practices, such as adding acid, salt and antimicrobial food additives, can lead to resistance against these microorganisms. (Gram & Dalgaard, 2002; Haute, Raes, Van der Meeren, & Sampers, 2016). Several alternatives have been developed to prolong the shelf-life of fish, by inhibiting or delaying the deterioration and the multiplication of microorganisms (Gómez-Estaca, Giménez, Gómez-Guillén, & Montero, 2010; Kakatkar, Gautam, & Shashidhar, 2017; Ojagh, Núñez-Flores, López-Caballero, Montero, & Gómez-Guillén, 2011). Particularly, bioactive films enable protecting foods and extending their shelf-life during storage and distribution (Atef, Rezaei, & Behrooz, 2015; Mohajer, Rezaei, & Hosseini, 2017; Rhim, 2013; Rocha, Loiko, Tondo, & Prentice, 2014; Salgado, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2013; Wu et al., 2014).

Conventional packaging is derived from petroleum and contributes to serious environmental problems (Arfat, Ahmed, & Jacob, 2017; Mohajer et al., 2017; Sousa & Gonçalves, 2015). Consequently, there is an increased interest in the development of bio-based and biodegradable films for food packaging (Atef et al., 2015; Gao et al., 2016; Gómez-Estaca et al., 2010; López de Lacey et al., 2014; Milkova & Radeva, 2015; Nuan mano, Prodpran, &
Films are thin layers of materials, which, once formed, can be placed on, or between, food components. These films contribute to enhancing the food shelf-life, by the incorporation of antimicrobial compounds or by providing an effective barrier to gases (CO₂, O₂), moisture and aroma, minimising mechanical damage (Atarés & Chiralt, 2016; Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). Natural biopolymers, such as proteins and polysaccharides, are suitable alternatives to petroleum-derived polymers, due to their desirable structural and mechanical characteristics and their biodegradability (Falguera et al., 2011).

Agar, an unbranched polysaccharide extracted from red algae, such as Gelidium and Gracilaria spp., is among the most attractive biopolymers for development of biodegradable films (Atef, Rezaei, & Behrooz, 2014; Mohajer et al., 2017; Rhim, Lee, & Hong, 2011). Films based on seaweed polysaccharides, such as agar, have excellent film-forming capability, good mechanical resistance and are edible and easily biodegradable (Atef et al., 2015; I. Choi, Lee, Lacroix, & Han, 2017; Rinaudo, 2008; Sousa & Gonçalves, 2015; Wang, Jo, An, Rhim, & Lee, 2015). Furthermore, agar forms biologically inert matrices, which facilitates the diffusion of various bioactive compounds to the food surface (The, Debeaufort, Voilley, & Luu, 2009). However, agar films have moderate water resistant properties (Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013).

Some essential oils (EOs) are generally recognised as safe (GRAS) by the Food and Drug Administration (FDA) and are considered as alternatives to the synthetic additives used in foods (Salgado et al., 2013). Clove (Syzygium aromaticum) EOs (CEOs) have been used in the elaboration of films, for fish conservation (Gómez-Estaca et al., 2010; Salgado et al., 2013). However, the use of EOs in foods may be limited, as they confer different flavours and aromas to the original food (Gómez-Estaca et al., 2010). The use of protein hydrolysates as food preservatives could be an alternative to overcome the inherent volatility and intense flavour of the essential oils. The peptides from hydrolysate are inactive within the sequences of the proteins, but might be released by enzymatic hydrolysis and may exert antimicrobial and antioxidant activities (Najafian & Babji, 2012). Some studies have evaluated the films with...
antioxidant properties by protein hydrolysate incorporation (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009; Salgado, Fernández, Drago, & Mauri, 2011). In addition, in recent years several studies reported on the antimicrobial activity of protein hydrolysates (PHs), particularly those derived from fish sources (Jemil et al., 2014; Kumar, Chatli, Singh, Mehta, & Kumar, 2016; Memarpoor-Yazdi, Asoodeh, & Chamani, 2012; Salampessy, Phillips, Seneweera, & Kailasapathy, 2010; Segura-Campos, Salazar-Vega, Chel-Guerrero, & Betancur-Ancona, 2013; Sila et al., 2014). However, no information is found about the incorporation of protein hydrolysates as antimicrobial agents into film forming solutions, which represent an important novelty in the present work. Alcalase is an endoprotease enzyme extract from *Bacillus licheniformis* while Protamex is an exoprotease and endoprotease complex from *Bacillus* (Choi, Hur, Choi, Konno, & Park, 2009). Thus, those enzymes are interesting to produce antimicrobial hydrolysates. The bioactive properties depend of amino acid composition, degree of hydrolysis and molecular weight profile of protein hydrolysates (Najafian & Babji, 2012). The antimicrobial effect of a PH is typically due to the fact of possessing low molecular weight (below 10 kDa) and less than 50 amino acids, of which, nearly 50% are hydrophobic (Najafian & Babji, 2012). This antimicrobial activity can be explained due to the hydrophobic portion and the positive charge of the hydrolysate, which binds to the anionic microbial surface, inducing membrane disruption (Chou et al., 2016).

The Argentine croaker (*Umbrina canosai*) is a fish available on the Southern coast of Brazil. It is underutilised, has low commercial value and a high protein content. The objective of this work was to valorize the Argentine croaker by obtaining and characterizing two hydrolysates using two types of enzymes: Alcalase and Protamex. The hydrolysate with the highest antimicrobial capacity was selected to be incorporated into agar films, which were compared to similar films containing clove essential oil. Both types of films were subsequently applied to flounder fillets and their effect during the chilled storage was evaluated.
2. Material and methods

2.1. Material

The Argentine croaker was obtained from the fishery industry of the city of Rio Grande, Southern Brazil in 2016, and processed at the Food Technology Laboratory of the Federal University of Rio Grande (FURG). Alcalase 2.4 L was provided by Novozymes Latin America (Araucária, Paraná, Brazil), and Protamex (1.5 AU/g) was purchased from Sigma–Aldrich (St. Louis MO, USA). Alcalase and Protamex specific activities of 13.3 and 14.0 U/mg protein, respectively, were determined by the method described by Cupp-Enyard (2008), which uses casein as substrate. All chemical reagents used in this research were analytical grade.

2.2. Preparation of Argentine croaker protein isolate

The Argentine croaker protein isolate (CPI) was obtained using the pH-shifting method, according to Undeland., Kelleher and Hultin (2002), with modifications. An aqueous dispersion of Argentine croaker muscle in distilled water at a ratio of 1:9 (w/v) was obtained, by solubilisation at pH 11.2 (1 M NaOH) at 4 °C for 20 min, under constant shaking on a propeller-shaker (Quimis, Q-251D2K, Campinas, Brazil). The samples were centrifuged at 9000 rpm (Hanil, Supra 22K, Korea) at 4 °C for 20 min. The precipitate was obtained by precipitation at pH 5.0 (1 M HCl) for 20 min at 4 °C under constant stirring, followed by centrifugation at 9000 rpm (Hanil, Supra 22K, Korea) at 4 °C for 20 min. The precipitate was lyophilised (Liotop L108, São Carlos, Brazil) at -55 °C and 50 µHg for 48 h, fractionated in a knife-mill (Tecnal, TE-633, Piracicaba, Brazil), sieved through a Nº. 42 mesh (0.35 mm) and stored at -18 °C. The CPI proximal composition was analysed, according to the method described by the Association of Official Analytical Chemists (AOAC, 2000), presenting 92.1% protein, 2.2% moisture, 1.2% lipids and 1.5% ash.
2.3. Preparation of Argentine croaker protein hydrolysate

The CPI was enzymatically hydrolysed as described by Liu et al. (2014) and Raghavan and Kristinsson (2009), with modifications. The CPI was suspended in distilled water in a 2% ratio (w/v; protein/distilled water) and homogenised at 300 rpm for 1 min using a homogeniser (Marconi, MA 259/MR, Piracicaba, Brazil). The homogenates were pre-incubated in a reactor (Marconi, MA 259/MR, Piracicaba, Brazil) at 50 °C and pH 8 for Alcalase and pH 7 for Protamex, respectively. The enzymatic hydrolysis was started by adding the enzyme Alcalase or Protamex in the proportion of 30 U/g protein (enzyme/substrate). The degree of hydrolysis (DH) was monitored using the pH-stat method (Adler-Nissen, 1986), until 20% DH was reached with each enzyme. The enzyme was inactivated by heating (Quimis, Q-215-1/2, Campinas, Brazil) the suspension at 90 °C for 10 min, followed by centrifugation (Hanil, Supra 22K, Korea) at 9,000 rpm at 4 °C for 20 min, to separate the soluble (hydrolysate) and insoluble fractions. The soluble fraction was freeze-dried (Liotop, L108, São Carlos, Brazil) and stored at -18 °C, until further use. Two hydrolysates with the same DH (20%) were obtained: A20 (by Alcalase hydrolysis) and P20 (by Protamex hydrolysis).

2.3.1. Amino acid composition

The amino acid composition of A20 and P20 hydrolysates was determined as described by Alemán, Giménez, Montero, and Gómez-Guillén (2011), with modifications. The hydrolysate was dissolved in distilled water (5 mg/mL) and 20 μL of sample was dried and hydrolysed in vacuum-sealed glass tubes at 110 °C for 24 h, in the presence of constant boiling 6 N HCl, containing 0.1% phenol and using norleucine (Sigma-Aldrich, St. Louis, MO, USA) as the internal standard. The hydrolysates were again vacuum-dried, dissolved in application buffer and injected into a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain). A standard mixture of amino acids was procured from Sigma-Aldrich (St. Louis, MO, USA).
Triplicate determinations were performed, and the results were expressed as the number of residues per 1000 residues.

2.3.2. Molecular weight distribution

For overall molecular weight (MW) distribution of A20 and P20 hydrolysates, size exclusion high-performance liquid chromatography (Shimadzu, SPE-MA10AVP, Kyoto, Japan) was conducted, according to Sila et al. (2015), using a Superdex Peptide PC 3.2/30 column (GE Healthcare Bio-Sciences, Barcelona, Spain), with a 100–7000 Da separation range. The hydrolysate (10 µL) was loaded onto the column and eluted at 2.5 µL/min flow rate, using acetonitrile (30%; v/v) with trifluoroacetic acid (0.01%; v/v) as the mobile phase. MW standards used were glycine (75 Da), hippuryl-L-histidyl-L-leucine (429 Da), vitamin B12 (1340 Da), aprotinin (6512 Da) and bovine serum albumin (6700 Da). Optical density was monitored at 214 nm.

2.3.3. Antimicrobial activity of protein hydrolysate

The antimicrobial activity of the A20 and P20 hydrolysates was measured, according to Gómez-Guillén, López-Caballero, Giménez, and Montero (2010), by the inhibition zone test against *Aeromonas hydrophila* CECT 839T, *Brochothrix thermosphacta* CECT 847, *Escherichia coli* CECT 515, *Debaryomyces hansenii* CECT 11364, *Listeria innocua* CECT 910, *Pseudomonas aeruginosa* CECT 110, *Shewanella putrefaciens* CECT 5346 CT, *Staphylococcus aureus* CECT 240, *Vibrio parahaemolyticus* CECT 511T and *Yersinia enterocolitica* CECT 4315. These strains were stored at -80 °C in brain heart (BHI) infusion broth (Oxoid, Basingstoke, UK) with 25% glycerol (Panreac, Moncada i Reixac, Barcelona, Spain), until use. The strains were grown in BHI broth, supplemented with 3% NaCl for *V. parahaemolyticus*. The microorganisms were incubated at 37 °C, except *A. hydrophila* and *S. putrefaciens*, which were incubated at 30 °C and *B. thermosphacta* at 25 °C. The antimicrobial activity was
measured on BHI spread plates inoculated with 100 μL of these microorganisms grown overnight (∼10⁶ CFU/mL). Then, sterile filter paper disks (0.5 cm diameter) soaked with 40 μl of hydrolysate (5 mg/mL) were laid onto the inoculated plate surface, followed by incubation under the conditions described above. Corel DRAW Graphics Suite X6 software was used to calculate the diameter of the clear inhibition zone around the disc. Triplicate determinations (mm) were performed.

2.4. Agar films preparation

The agar films were prepared by a casting technique, as described by Giménez et al. (2013) and Salgado et al. (2013), with modifications. The hydrolysate displaying the most effective antimicrobial activity (as described in 2.3.3 item) was chosen to elaborate the active films, as follows: the film-forming solution (FS) was prepared by dispersing 1 g agar in 100 mL of distilled water at 90 °C for 30 min, under continuous agitation by magnetic stirring. Glycerol was used as plasticiser at 30% (g of glycerol/100 g agar) and homogenised for 20 min under constant agitation by magnetic stirring. Based on preliminary studies of antimicrobial capacity, the PH (0.5 g PH/g agar) was added and homogenised for 20 min, under continuous stirring. For comparison purposes, the CEO (Petite Marie, 010742PM, São Paulo, Brazil) was added at the same concentration (0.5 g CEO/g agar). As a control, an agar film without the addition of PH or CEO was used. Each FS, having the same solid content (0.0053 g/cm²), was added to acrylic Petri dishes (9 cm diameter) and then oven-dried with forced air circulation at 37 °C for 16–18 h. Films were conditioned in desiccators containing saturated solutions of sodium bromide (NaBr) at 22 °C and 58% relative humidity (RH) for 48 h. As a control, a film without the addition of PH or CEO was used.

2.4.1. Thickness
The film thickness was determined using a digital micrometer, with 0.001 mm resolution (Insize, IP54, São Paulo, Brazil). Measurements were performed at ten random locations on the surface, according to Gontard, Guilbert, and Cuq (1992).

2.4.2. Mechanical properties

The tensile strength (TS; MPa) and elongation at break (EB; %) of the films, were assessed, as described by the American Society for Testing and Materials method ASTM D882-91 (ASTM, 1996) using a texture analyser (TA.XT plus, Stable Micro Systems, Haslemere, England). The films were cut into rectangles (80 mm × 25 mm), and initial grip separation and crosshead speed were set at 50 mm and 0.8 mm/s, respectively. TS and EB were measured on nine strips of films, from which three triplicates were obtained.

2.4.3. Water vapour permeability (WVP)

The WVP was measured using a method described by Sobral, Menegalli, Hubinger, and Roques (2001), with modifications. The film was sealed in a cell with a permeation area of 15.90 cm² containing dry silica (0% RH) and placed in a desiccator with distilled water (100% RH) at 22 °C. The cells were weighed every hour for 7 h, and results were expressed as g mm/ h cm² Pa. All tests were performed in triplicate.

2.4.4. Film solubility

Water solubility of the film samples was determined according to the method described by Gontard, Guilbert, and Cuq (1992). The films were cut into squares (2 × 2 cm²), oven-dried (Fanem, 520, São Paulo, Brazil) at 105 °C for 24 h, and placed in plastic containers with 15 mL of distilled water at 22 °C for 24 h. The solution was then filtered through Whatman n°.1 filter paper, to recover undissolved film, which was dried at 105 °C (Fanem, 520, São Paulo, Brazil) for 24 h. The weight of solubilised dry matter was calculated, by subtracting the weight of
undissolved dry matter from the initial weight of dry matter and expressed as the percentage of

total weight.

2.4.5. Colour, opacity and transparency

The colour was determined in triplicate using a colourimeter (Minolta, CR-400, Osaka, Japan), based on the CIELAB colour space (CIE, 1986). Measurements were performed on white standard backgrounds (luminosity, $L^* = 97.75$; greenness-redness, $a^* = -0.49$ and blueness-yellowness, $b^* = 1.94$), where $L^*$ ranges from 0 (black) to 100 (white); $-a^*$ (green) to $+a^*$ (red); and $-b^*$ (blue) to $+b^*$ (yellow).

Opacity ($Y$) of the samples was determined in triplicate, using a colourimeter (Minolta, CR-400, Osaka, Japan), according to Sobral, Menegalli, Hubinger, and Roques (2001), with adaptations. The opacity of the film samples was based on the association between the opacity of each sample on the black standard ($Y_b$) and the opacity of each sample on the white standard ($Y_w$).

The transparency was evaluated in a spectrophotometer (Bioespectro, SP22, São Paulo, Brazil) at 600 nm, as described by Atef et al. (2014) and Han and Floros (1997), using the following equation:

$$\text{Transparency} = \frac{Abs\ 600}{x}$$

where $Abs\ 600$ is the absorbance measured at 600 nm, and $x$ is the film thickness (mm). Less transparent films have higher transparency values.

2.4.6. Microstructure of the films
Scanning electron microscopy (SEM; Jeol, JSM-6610LV, Tokyo, Japan), operating at 10 kV, was used to evaluate the surface and freeze-fractured cross-section morphology of the films (PH, CEO and control). For cross-section, samples were fractured under liquid nitrogen prior to morphology visualization. Samples were cut and deposited on aluminium stubs using double-sided tape, and gold-sputtered (Desk, Denton Vacuum, USA) for 120 s. Images were captured at 1000× magnification for surface and at 500× magnification for freeze-fractured cross-section morphology.

2.5. Effect of films on preservation of flounder fillets

Flounder (*P. orbignyanus*) (Rio Grande, Brazil) captured at Patos Lagoon estuary in November 2016 and filleted by professional operators, were purchased at a local market and transported at 4 °C, to the Laboratory of Food Technology of the Federal University of Rio Grande, within 20 min. The flounder fillets were composed of 80.4% moisture, 18.5% protein, 1.4% lipids and 0.7% ash, determined according to the method described by the AOAC (2000).

For the storage trial, the flounder fillets were cut into rectangular portions of approximately 30 g (5 × 4 cm²) and randomly separated into three batches. The fillets were wrapped with two films (9 cm in diameter), in which all sides of the samples were completely covered with one film and manually sealed. According to the film nomenclature described in section 2.4, several fish batches were studied: fillets covered with PH films (PH film), fillets covered with CEO films (CEO film) and fillets covered with agar films (Control film) for control purposes. Next, fish fillets were placed on polystyrene trays and stored at 5 ± 1 °C (Marconi, MA 415/S, Piracicaba, Brazil) for 15 days. The covered fillets were evaluated periodically (0, 3, 5, 7, 10 and 15 days) for pH, total volatile basic nitrogen (TVB-N), weight loss and microbiological analyses. Fish fillets (*P. orbignyanus*) covered were aseptically peeled before each analysis.

2.5.1. pH
A fillet aliquot (10 g) was homogenised in distilled water at 1:2 (w/v) ratio. Then, the pH was determined in triplicate (Quimis, Q400AS, Campinas, Brazil), after 5 min at room temperature, based on the method described by Gómez-Estaca et al. (2010).

2.5.2. TVB-N

The TVB-N of the fillets was analysed, as outlined by the AOAC (1990), with modifications. Briefly, 25 g of the ground fillet was weighed in a suitable container and homogenised with 40 mL of 7.5% (w/v) trichloroacetic acid (TCA) for 1 min. Next, the mixture obtained was vacuum filtered through Whatman n°. 1 paper, washed with 5 mL of 7.5% (w/v) TCA and adjusted to 50 mL. The filtrate (10 mL) was distilled (Tecnal, TE-036/1, Piracicaba, Brazil) and the distillate was collected on boric acid (50 g/L) and, then, titrated with hydrochloric acid (0.02 M HCl). Analyses were performed at least in triplicate. The results were expressed as mg TVB-N/100 g sample.

2.5.3. Weight loss

The weight loss was determined gravimetrically in triplicate, as described by Han, Wang, Li, Lu, and Cui (2014). The weight loss was expressed as a percentage of the original weight of the covered fillets.

2.5.4. Microbiological analyses

The antimicrobial effect of the various films during storage was evaluated according to the methods described by Arancibia, López-Caballero, Gómez-Guillén, and Montero (2015) and Salgado et al. (2013). Fish fillets (P. orbignyanus) were aseptically peeled and 10.0 ± 0.2 g transferred to sterile bags (Interscience, Saint-Nom-la-Bretèche, France) containing 90 ml of 0.1% (w/v) sterile peptone water (Himedia, Mumbai, India), followed by homogenisation in a
Stomacher blender (ITR, MR 1204, Esteio, Brazil) at room temperature (25 °C) for 1 min.

Then, appropriate dilutions were prepared for determination of (i) total aerobic mesophiles on pour plates of plate count agar (Oxoid, Basingstoke, UK), incubated at 30 °C for 72 h; (ii) H₂S-producing organisms, as black colonies, on spread plates of iron agar (Microkit, Madrid, Spain) incubated at 15 °C for 3 days; (iii) *Pseudomonas* spp. on spread plates of *Pseudomonas* agar base (Difco, Bordeaux, France) incubated at 25 °C for 48 h; (iv) *Enterobacteriaceae* on double-layered plates of violet red glucose agar (Acumedia, Lansing, Michigan, USA), incubated at 30 °C for 48 h, and (v) lactic acid bacteria, on double-layered plates of Mann–Rogosa–Sharpe agar (Kasvi, Italy) incubated at 30 °C for 72 h. The microbiological counts were performed in triplicate and expressed as log of colony-forming units per gram sample (log cfu/g).

2.6. Statistical analysis

All the results, except the MW distribution, were evaluated by analysis of variance (ANOVA). The means were compared by Tukey’s test at 5% significance level and assessed by Statistica version 5.0 (StatSoft, Inc., Tulsa, USA).

3. Results and discussion

3.1. Characterization of protein hydrolysate

The amino acid composition of PHs influences their bioactive properties (Di Bernardini et al., 2011; Najafian & Babji, 2012). For both A20 and P20 hydrolysates, the major amino acids were aspartic acid (Asp), glutamic acid (Glu), alanine (Ala), leucine (Leu) and lysine (Lys) (Table 1). A very similar amino acid composition was observed in several previous studies with PHs derived from marine sources (Je, Park, Hwang, & Ahn, 2015; Najafian &
The presence of hydrophobic amino acids (HAAs), such as Ala, valine (Val), isoleucine (Iso), Leu, tyrosine (Tyr), phenylalanine (Phe), proline (Pro), methionine (Met) and cysteine (Cys), has been shown to enhance the potency of antimicrobial peptides (He, Girgih, Malomo, Ju, & Aluko, 2013). In this study, A20 showed a higher HAA content, with 385.25 residues/1000 residues, than P20 (374.78 residues/1000 residues) (p < 0.05). Alcalase has been reported to preferably hydrolyse HAAs (Intarasirisawat, Benjakul, Wu, & Visessanguan, 2013). Some authors (Cheng, Tang, Wang, & Mao, 2013; He et al., 2013) stated that the interaction between antimicrobial peptides and the bacterial cell membrane could be associated with the HAAs, due to the formation of hydrophobic bonds. Hydrolysates with cationic properties offer antimicrobial activity against many pathogenic microorganisms, such as Gram-negative and Gram-positive bacteria (Agyei & Danquah, 2011). In the current study, the amino acid content revealed no significant difference (p > 0.05) in the positively-charged amino acid content (arginine (Arg), histidine (His), Lys) between both hydrolysates.

The overall MW distribution of A20 (Fig. 1a) and P20 (Fig. 1b) depended on the enzyme used, as expected (Alemán et al. 2011). The A20 polypeptide had an MW of about 1083 Da, whereas the hydrolysates obtained with Protamex (P20) contained peptides with MW below 1350 Da. Unlike Protamex, Alcalase does not require activation by a metal ion, and this could be associated with its higher hydrolytic activity (Klompong, Benjakul, Kantachote, & Shahidi, 2007). In general, antimicrobial peptides are relatively small molecules and have a broad spectrum of antimicrobial activity (Maturana et al., 2017; Najafian & Babji, 2012; Sila et al., 2014).

The antimicrobial activity of the hydrolysate samples (5 mg/mL) was evaluated by the agar diffusion method, based on the clear zone of inhibition surrounding the circular discs. As shown in Table 2, for the 10 strains evaluated, only six were inhibited. A20 did not inhibit the growth of Gram-negative bacteria (E. coli, P. aeruginosa, S. putrefaciens and V. parahaemolyticus). However, this hydrolysate exhibited a higher inhibition halo (14.52 mm) against foodborne Gram-positive bacteria, such as S. aureus, followed by the yeast D. hansenii.
(10.87 mm) (p < 0.05), than P20. Furthermore, some bacteria, such as *A. hydrophila*, *L. innocua* and *Y. enterocolitica*, presented sensitivity only to A20. The differences in membrane composition of the microorganisms have implications for their mechanism of action and the specificity of the antibacterial substance. The complex structure of the Gram-negative bacterial cell wall acts as a barrier that prevents the bacteria from the active compounds (Graef et al., 2016; Tenover, 2006). The first barrier for the antimicrobial peptides acting on Gram-negative bacteria is the lipopolysaccharide membrane. After the deterioration of this membrane, the peptide activity depends on its ability to interact with the cytoplasmic membranes. The amino acid sequence and concentration of the peptide, as well as the composition of the bacteria membrane, influence the mechanism of interaction (Gómez-Guillén et al., 2010).

The differences in the antimicrobial activities of A20 and P20 might be attributed to the differences in their molecular structure (MW, amino acid composition and sequence) and peptide length (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Najafian & Babji, 2012; Wald et al., 2016). The A20 peptides have an overall MW of 1083 Da, as shown in Fig. 1. According to Beaulieu, Thibodeau, Bonnet, Bryl, and Carbonneau (2013), peptides below 10 kDa possess improved antibacterial activity. The MW might be related to the elimination of aggregates and higher exposure of the amino acids and their charges, facilitating the interaction with bacterial membranes (Gómez-Guillén et al., 2010). Ennaas, Hammami, Beaulieu, and Fliss (2015) studied the hydrolysate of Atlantic mackerel (*Scomber scombrus*) by-products and, similarly to the present study, showed the low MW hydrolysates were effectively active against Gram-positive and Gram-negative bacterial strains. However, Wald et al. (2016) evaluated pepsin hydrolysed trout viscera, at 10, 20, 25 and 30% DH, and demonstrated that after 25% DH, the hydrolysates demonstrated no significant difference (p > 0.05) in their antibacterial activity.

Cheng, Tang, Wang, and Mao (2013) suggested that the hydrophobicity of peptides facilitates their entry into the membrane, as the positive charge would initiate the peptide interaction with the naturally negatively-charged surface of the bacteria. Additionally, Hancock and Scott (2000) explained that hydrophobic portions of the PHs are used for their insertion into
the cell membrane of the microorganism, thus, destabilising the cellular water balance and causing cell lysis. The A20 peptides had a higher HAA content (p < 0.05) than P20, as shown in Table 1. Wald et al. (2016) and Robert et al. (2015) reported that peptides with higher hydrophobicity possess higher antimicrobial activity than those with lower hydrophobicity. The results of the present work indicated that the antibacterial peptides derived from A20 showed higher HAA content, which is an attribute that makes them potential candidates for application as natural antimicrobial agent.

3.2. Effect of hydrolysate incorporation on properties of the films

Agar films without active compounds (control) and with PH were more transparent and homogeneous than films with CEO. In the CEO films, slight lipid exudation induced the presence of small lipid droplets on the films surface, indicating that the amount of added oil made somewhat difficult the homogenisation of the agar film forming solution. On the other hand, the hydrolysate did not dissolve in the presence of oil, therefore these compounds could not be tested together in the same formulation. In previous studies, the addition of EOs also affected film appearance by roughness or lipid droplets, throughout the film (Altiok, Altiok, & Tihminlioglu, 2010; Sanchez-Gonzalez, Vargas, Gonzalez-Martínez, Chiralt, & Chafer, 2009).

3.2.1. Film thickness and mechanical properties

The effects of the incorporation of CEO or PH on the properties of the agar films are presented in Table 3. The thickness of the control (0.043 mm) and the PH (0.044 mm) films showed no significant difference (p > 0.05). The addition of CEO resulted in a significant increase (p < 0.05) in film thickness (0.061 mm), probably due to a less compact matrix formation by certain incompatibility between agar and CEO. Atef et al. (2015) reported that the thickness of agar-cellulose bionanocomposite films incorporated with summer savory (Satureja
hortensis) EO significantly increased (p < 0.05) from 0.073 to 0.088 mm, with increased EO added to the film-forming solution.

The mechanical strength and EB are important properties of packaging films, to maintain their integrity and tolerate external stress (Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015). The influence of added active compounds on the mechanical properties (TS and EB) of the films, is shown in Table 3. The TS and EB of the control films were 27.46 MPa and 22.24%, respectively. However, the addition of PH or CEO caused a significant decrease (p < 0.05) in TS, to 19.89 and 10.16 MPa, respectively. Atef et al. (2015) documented that agar-cellulose bionanocomposite films incorporated with summer savory (S. hortensis) EO showed a decrease in TS from 31.21 MPa (control) to 28.26 MPa, with the addition of 0.5% EO. Several studies have reported a reduction in TS, caused by the addition of EOs (Atef et al., 2015; Do Socorro Rocha Bastos et al., 2016; Klangmuang & Sothornvit, 2016). The primary explanation for this effect could be explained as hindrance of polymer chain-to-chain interactions and improved flexible domains within the film, by the addition of oil in polysaccharide-based films. Furthermore, a weakening effect is caused, mainly attributed to the heterogeneous biphasic structure caused by lipid addition (Atarés & Chiralt, 2016).

Nuanmano et al. (2015) informed that short chain peptides could act as plasticisers in protein films, reducing the interaction between the polymers, increasing the free volume between them and reducing the EB and TS, as verified in the current study. The films incorporated with hydrolysate presented a significant increase (p < 0.05) in the EB (42.70%). However, this effect was not observed on the CEO films, which presented an EB of 3.93%. Similar results were published by Atef et al. (2015), who noted that the EB of agar-cellulose bionanocomposite films decreased with the addition of summer savory (S. hortensis) EO. A previous study by Altiok et al. (2010) confirmed the strong influence of thyme (Thyme vulgaris) EO on the EB, by adding 0 to 1.2% EO because the films showed a decrease in EB from 4.8 to 1.8%. As presented in Table 3, the addition of CEO induced a significant decrease (p < 0.05) in the EB. Such behaviour occurred because a heterogeneous and irregular structure caused by exudation of CEO was observed in the obtained film matrix.
Barrier properties of films, such as WVP, are important parameters for estimating the packaged product shelf-life and food packaging applications (Kanatt, Rao, Chawla, & Sharma, 2012). The WVP and solubility of the films samples are presented in Table 3. Films incorporated with PH (3.61 g mm/h Pa cm²) had a higher WVP than control films (1.40 g mm/h Pa cm²) (p < 0.05). Similar results were reported by Giménez, Alemán, Montero, and Gómez-Guillén (2009) and Nuanmano et al. (2015), who found that the addition of PHs resulted in an increased WVP of films elaborated with various matrices. Cuq, Gontard, Cuq, and Guilbert (1997) stated that a plasticiser, such as glycerol, can modify the three-dimensional molecular organisation of the film matrix, decreasing the intermolecular attractive forces and increasing the free volume and chain mobility, and thereby, enhancing the diffusion rate of WVP. Accordingly, in the current study, due to their plasticising effect, the addition of the PHs increased the EB and WVP of the films. A20 peptides have a low overall MW distribution (1083 Da) and may contain a large number of hydrophilic groups (-NH₂, -COOH, -OH). Thus, adding A20 to the film-forming solution causes the films to absorb a substantial number of water molecules (Nuanmano et al., 2015). Agar films incorporated with CEO (3.37 g mm/h Pa cm²) showed an increased in WVP compared with control films (p < 0.05). These results concur with previous studies (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Altıok et al., 2010; Atef et al., 2015). Based on Atarés, De Jesús, Talens, and Chiralt (2010), the effect of EO addition on film microstructure is a determinant factor, to modify the water barrier properties. In the present study, it was possible to visibly verify the presence of oil exudation on the film surface, forming preferred routes for the diffusion of water vapour, resulting in an increase in WVP.

Solubility is considered an indicator of the resistance of the film sample to water. For food packaging applications, where water activity is high, or when the film must be in contact with water and act as a food protector, a low film solubility is necessary (Atef et al., 2015;
In the present study, the CEO films presented noticeable lower solubility (20.86%) than the PH film (48.86%) (p < 0.05), in agreement to the highly hydrophobic nature of the essential oil. Giménez et al. (2013) reported that agar films showed a higher solubility (24.1%) for films elaborated with 1.5% agar (w/v) than that observed in the present study (21.95%). This effect may be due to the superior agar content used in the elaboration of the polymeric matrix. Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, and Montero (2009) elaborated squid (Dosidicus gigas) skin gelatin films with the addition of hydrolysates from squid gelatin and verified no significant differences (p > 0.05) in solubility by addition of 0 to 10% PH. Nuanmano et al. (2015) suggested that an increase in film solubility by hydrolysate incorporation may occur due to weak interactions between short chain peptides and the polymer matrix.

Wu et al. (2014) incorporated oregano EO (0, 1, 2 and 3%) into silver carp (Hypophthalmichthys molitrix) skin gelatin-chitosan films and did not describe a significant increase (p > 0.05) in film solubility. Atef et al. (2015) demonstrated that agar films incorporated with 0 and 0.5% summer savory (S. hortensis) EO, had a similar solubility of 29.68 and 29.67%, respectively. Some authors (Ahmad et al., 2012; Ojagh, Rezaei, Razavi, & Hosseini, 2010) reported that incorporation of EO might result in a decrease in film solubility, due to the hydrophobic nature of oil and a good interaction between the polymer matrix and EO. However, those authors worked with highly soluble film matrices made of gelatin or chitosan. The current study did not show a significant decrease in film solubility with CEO incorporation (20.86%) because the control agar film was already highly insoluble (21.95%).

### 3.2.3. Optical properties

Optical properties of films, such as colour, opacity and transparency, are important attributes that influence their appearance, acceptance, commercialisation, marketability, and their suitability for various applications (Ahmad et al., 2012). The optical properties of the control, CEO and PH films, are shown in Table 3. The results indicate that the addition of PH
and CEO did not show a significant difference in luminosity ($L^*$) of the control films, which showed a greater tendency to a light colour. The same behaviour was observed in several previous studies, such as Atef et al. (2014), Pires et al. (2013) and Pranoto, Salokhe, and Rakshit (2005). The PH films showed $b^*$ (4.21) and $a^*$ (-0.61) values significantly different to the CEO and control films ($p < 0.05$). The tendency to yellowness with hydrolysate additions was also verified by Nuanmano et al. (2015) in protein films. According to these authors, the yellowness is due to the amino groups (-NH$_2$) of the hydrolysate, which may interact with carbonyl groups (C=O) of lipid oxidation products in a polymeric matrix, via the Maillard reaction, particularly during drying of the film. Atef et al. (2015) observed that the addition of 0.5% EO to agar-cellulose bionanocomposite films increased the yellowness ($b^* +$) of the films, from $b^*$ 5.00 to 7.68, with the EO addition, but this was not verified in the current study.

The opacity values of the control, CEO and PH films, are shown in Table 3. The control and PH films did not present a significant difference ($p > 0.05$) in opacity. However, CEO films were significantly more opaque ($Y = 12.80$) compared to the other films elaborated ($p < 0.05$). Do Socorro Rocha Bastos et al. (2016) found that cellulose acetate films, incorporated with rosemary pepper (Lippia sidoides) and basil (Ocimum gratissimum) EOs resulted in a higher opacity than the films without EO addition. In the present study, the highest thickness (0.061 mm) observed for CEO films, may have contributed to their increased opacity.

PH films ($T = 1.55$) did not show significant difference ($p > 0.05$) in transparency relative to the control films ($T = 1.57$). According to Nuanmano et al. (2015) and Giménez et al. (2009), the shorter hydrolysate peptides, with a higher number of chain ends, may act as an effective plasticiser, by preventing polymer-polymer interactions, leading to a less dense film matrix with more transparency. Nevertheless, the addition of CEO yielded films with decreased transparency (4.87). Teixeira et al. (2014) found that CEO-incorporated protein films also showed decreased transparency when compared to control films. According to Atarés and Chiralt (2016), the incorporation of EO results in a decrease in light transmission that may have been due to dispersion at the interface of the oil droplets embedded in the film matrix.
3.2.4. SEM

SEM micrographs of the surface and cross-section of films from agar films incorporated with PH and CEO are illustrated in Fig. 2. The control films (Fig. 2a) appeared to exhibit the formation of aggregates, possibly between glycerol and agar, due to the presence of large areas of crosslink density, which may have contributed to the higher TS observed for this film. Likewise, González, Strumia, and Igarzabal (2011) also verified that the formation of aggregates between the polymeric matrix and glycerol presents a change in the SEM images. The control film had a compact cross-section (Fig. 2b) without pores or cracks. SEM image of PH films (Fig. 2c) revealed a smooth surface. Some regions had small particles, which may have been the added hydrolysate, contributing to the increased WVP of the film, as shown in Table 3. Furthermore, the cross-section of PH films (Fig. 2d) was smoother and denser than the control film. However, this cross-section showed cracks which contribute to WVP enhancement. The cross-section of the CEO film (Fig. 2f) became less rougher compared to control films. The CEO film micrograph (Fig. 2e) showed roughness and oil droplets on the surface. These regions can contribute to WVP enhancement, by acting as preferred routes for the diffusion of water vapour, as verified in Table 3. These results indicate the incompatibility between CEO and the polymeric matrix, resulting in a discontinuous structure. In agreement with these results, Altiok et al. (2010), Atef et al. (2014) and Wu et al. (2014) observed the same behaviour for various polymer films incorporated with EOs.

3.3. Preservation of flounder fillets

In the last decades, the application of antimicrobial food packaging has received substantial research interest (Arancibia et al., 2015; Atarés & Chiralt, 2016; Mulla et al., 2016; Rocha et al., 2014; Yu, Jiang, Xu, & Xia, 2017). Although there is increasing literature available regarding the development of active packing films, there are limited studies about the antimicrobial properties of fish PH from Argentine croaker on the shelf-life of fish fillets.
Considering the *in vitro* antimicrobial properties of A20, the next step was to evaluate the efficacy of this hydrolysate incorporated in agar film on the preservation of flounder fillets, by comparing with a control film (agar film) and an analogous film made with addition of clove essential oil. It should be noted that the Alcalase hydrolysate did not present strong undesirable fishy smell or bitter taste. Thus, the flavour of flounder fillets was not perceptibly modified by the agar films containing the fish protein hydrolysate. In contrast, the clove essential oil provided a slight characteristic odour during the storage. It should be also noted that the agar films presented very good mechanical resistance when applied to the fillet surface, resulting from their relatively low water solubility. Even the PH film, which showed significantly higher solubility, could be easily peeled from the fish without losing its structural integrity.

### 3.3.1 pH and TVB-N

The changes in biochemical parameters, such as pH and TVB-N of flounder fillet, are shown in Fig. 3. The flounder fillets had an initial pH of 6.64 (Fig. 3a), and this value significantly increased (p < 0.05) to pH 7.11, during the cold storage of the samples covered with control films. Except for PH films, pH slightly decreased within the first 3 days, and then increased during storage. Similar results had been observed previously (López de Lacey et al., 2014; Yu et al., 2017). This behaviour occurred due to the liberation of inorganic phosphate by the degradation of adenosine triphosphate (ATP) and accumulated lactic acid during anaerobic glycolysis, caused by the rigor stage of the fish (Liu et al., 2013). For the flounder fillet covered with PH film, the pH increased significantly (p < 0.05) to 7.05 after 15 days of storage. However, for the sample coated with the control and CEO films, a significant increase (p < 0.05) to pH 7.11 and 6.76 was observed up to 10 days of storage, respectively. According to Seabra et al. (2011) and Salgado et al. (2013), the increase in pH throughout storage might be attributed to accumulation and formation of alkaline compounds, such as biogenic amines and ammonia, produced by the action of microorganisms and endogenous enzymes. Compared to the other treatments, a lower pH value (p < 0.05) was observed in flounder fillets covered with
CEO films, thus, suggesting that CEO films contributed to reducing the formation of alkaline compounds.

The TVB-N values obtained for the samples under study are shown in Fig. 3b. A significant increase (p < 0.05) in TVB-N content from 8.35 to 33.97 mg TVB-N/100 g was verified, for fillets covered with control films on day 15. However, for the samples coated with PH or CEO films, there was a significant increase (p < 0.05) from 8.35 to 27.72 and 25.39 mg TVB-N/100 g sample until day 10, respectively, accounting for the increases in pH during the storage period (Fig. 3a). After 15 days of storage, the samples covered with PH and CEO films presented 29.80 and 25.83 mg TVB-N/100 g sample, respectively. Wu et al. (2014) detected that grass carp (Ctenopharyngodon idellus) muscle fillets packaged with gelatin-chitosan films incorporated with oregano EO during storage at 4 °C for 12 days, had 40 mg TVB-N/100 g sample, which is higher than that verified in the present study. The increase in the TVB-N content during storage has been confirmed in several studies (Arancibia, López-Caballero, Gómez-Guillén, & Montero, 2014; Gómez-Estaca et al., 2010; Lekjing, 2016). According to Ojagh et al. (2011), the established limit for fresh fish of 30 mg TVB-N/100 g muscle was only exceeded by the control films. However, it should be noted that the CEO and PH in agar films were only efficient to fish freshness in the last stages of chilled storage, i.e. from day 7 and day 10 with the CEO-film and PH-film, respectively.

3.3.2. Weight loss

The weight loss of all fish fillets increased significantly (p < 0.05) throughout the storage (Fig. 4), being mainly attributed to a decrease in water holding capacity of the muscle protein, causing water to exudate. Part of the drip water was trapped in the agar films which could swell slightly, but did not lose integrity, thus contributing to an overall improvement in visual appearance. The weight loss was significantly higher (p < 0.05) for fillets covered with agar films incorporated with hydrolysate and CEO than the control film at the day 15 of storage, displaying a mass loss of 54.22 and 52.14%, respectively. These results indicated that the
structural changes induced by PH or CEO in the film matrix might have increased the affinity of
agar by water molecules, thus favouring some protein dehydration. In contrast to this finding,
Han et al. (2014) observed that beef fillets covered with polypropylene and polyvinyl alcohol
films incorporated with different EOs did not show a significant weight loss relative to the
control film during storage.

The incorporation of hydrolysate enhanced the EB of the film. Furthermore, it led to a
decrease in the films barrier properties. This behaviour occurs because the short chain peptides
act as plasticisers in films, reducing the interaction between the polymers, consequently,
increasing the free volume for gas or water molecules to diffuse through the polymer matrix
(Nuanmano et al., 2015). Such an effect is important in food packaging, due to the role played
by water in deteriorative reactions and microbial growth (Jamshidian, Tehrany, Imran, Jacquot,
& Desobry, 2010). In the present study, the CEO and PH films displayed a higher WVP than the
control film, due to the microstructural and physicochemical characteristics of the antimicrobial
films. This result is attributed to the greater weight loss of the treated fillets compared to the
control.

3.3.3. Microbiological analyses

The microbial counts of flounder fillet covered with the various films are shown in Fig. 5. At the beginning of the storage period, the counts of Enterobacteria (Fig. 5a) were about 2
log cfu/g. This figure was lower than 3.19 log cfu/g found in olive flounder (Paralichthys
olivaceus) reported by Li et al. (2017). The total aerobic mesophiles (Fig. 5b) and H2S-
producing microorganisms (Fig. 5c), such as black colonies, possibly Shewanella putrefaciens,
were around 2 and 3 log CFU/g, respectively. The initial number of Pseudomonas spp. was 3.4
log CFU/g (Fig. 5d). Pseudomonas spp. and H2S-producing bacteria, are specific spoilage
organisms in fish or fish products, during chilled storage. The flounder fillets used in this study,
were of high quality, as indicated by a low initial microbial count (less than 3.5 log CFU/g).
In general, the microbial counts increased through storage, in all evaluated treatments (p < 0.05). However, agar films incorporated with CEO did not show a significant increase in H₂S-producing microorganisms count after the day 3, remaining practically constant until the end of the period (Fig. 5c). Furthermore, these films inhibited (p < 0.05) total aerobic mesophiles (Fig 5b), lactic acid bacteria (Fig. 5e) and H₂S-producing microorganisms (Fig. 5c), approximately 1.5, 1.3 and 2.15 log CFU/g, respectively, at the end of storage. Apparently, the CEO in agar films inhibited bacterial activity, due to the hydrophobic nature of the oil and its constituents, such as phenolic compounds (i.e., carvacrol, eugenol and thymol). These characteristics result in a partition in the lipids of the bacteria cell membrane and mitochondria, thereby, disturbing such structures and enhancing cell permeability and cytoplasm loss. Furthermore, those compounds can interact with enzymes present in the cell wall, leading to lesions and decreasing cellular ATP production and intracellular pH, resulting in the loss of cell viability (Lekjing, 2016; Mulla et al., 2016; Sikkema, De Bont, & Poolman, 1994; Wu et al., 2014; Zivanovic, Chi, & Draughon, 2005). Another related study, Gómez-Estaca et al. (2010) demonstrated that cod fillets (Gadus morhua) coated with gelatin-chitosan films incorporated with CEO, completely inhibited H₂S-producing microorganisms, after 3 days of storage, which may be related to the decrease in pH caused by the active film.

The A20 hydrolysate, incorporated in agar films, showed the highest HAA content and the lowest MW, as shown in Table 1 and Fig. 1, that contributed to insertion of the peptides into the cell membrane (Najafian & Babji, 2012; Segura-Campos et al., 2013). The PH films reduced the H₂S-producing microorganisms (Fig. 5c), lactic acid bacteria (Fig. 5e) and total aerobic mesophiles (Fig 5b) by 0.94; 0.54 and 0.21 log CFU/g, respectively, compared to the control films. However, the PH films presented a lower inhibition of the microorganism groups evaluated and higher pH and TVB-N values than the CEO film (Fig. 5).

The final *Pseudomonas* spp. counts (Fig. 5d) did not show a significant difference (p > 0.05) for all treatments evaluated on the day 15 of storage, however, a notable reduction was observed with the CEO-film from day 7 to 10. Respecting to *Enterobacteria*, the inhibitory effect of PH and CEO films is lost as conservation progresses. Salgado et al. (2013) recorded...
high values of this bacteria (8.79 log CFU/g) in sardine (S. pilchardus) patties coated by a gelatin-chitosan film incorporated with CEO at the end of the chilled storage. According to some authors (Atef et al., 2015; Sedaghati, Ezzatpanah, Akbar, Ebrahimi, & Kobarfard, 2016; Wu et al., 2014), Gram-negative bacteria, such as Enterobacteria (Fig. 5a) and Pseudomonas spp. (Fig. 5d), are more resistant, due to a relatively impermeable cell membrane, limiting the diffusion of hydrophobic compounds, such as CEO and PH. This finding concurs with the antimicrobial activity tested in this study (Table 2), where the PH did not show inhibition against as E. coli and P. aeruginosa. The increase in TVB-N and pH values of the different fish fillets showed a similar increasing tendency to the total aerobic mesophiles, Enterobacteria and Pseudomonas spp., of around 8 log CFU/g, for the fillets covered with PH and control films.

The addition of CEO and PH in agar films did not present effective inhibition of some microorganisms evaluated, compared to other studies with active films, such as Gómez-Estaca et al. (2010), Han et al. (2014) and Wu et al. (2014). However, the CEO and PH films could extend the shelf-life of flounder fillets by improving biochemical and microbiological parameters in the last stages of the studied chilled storage. In general, CEO films were found more effective than PH films, but both films were shown particularly effective at inhibiting the growth of H₂S producing microorganisms from day 3 onwards.

4. Conclusion

The Alcalase hydrolysate was found more effective against selected microorganisms than the Protamex hydrolysate, therefore it was employed to provide an agar film with antimicrobial activity. The efficacy of this film on the preservation of flounder fillets was slightly lower than with an analogous film made with addition of clove essential oil, a well-recognized antimicrobial agent. However, both types of film contributed to extend the shelf-life of flounder fillets by improving biochemical and microbiological parameters in the last stages of the chilled storage. Despite the significant increase in water solubility caused by the hydrolysate addition, the agar-PH film presented higher mechanical properties and transparency than the
CEO film, which make it suitable for fish packaging without the sensory limitation of the essential oil volatile compounds. Further studies would be necessary to improve the antimicrobial capacity of this film by favouring the controlled release of peptides when used as food packaging.

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antimicrobial activity of silver carp (*Hypophthalmichthys molitrix*) skin gelatin-chitosan
films incorporated with oregano essential oil for fish preservation. *Food Packaging and
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(*Ctenopharyngodon idellus*) fillets by chitosan coating combined with glycerol

Table 1. Amino acid composition of hydrolysates

A20: hydrolysate by Alcalase with a 20% DH; P20: hydrolysate by Protamex with a 20% DH; Lower case in the same row indicate significant (p < 0.05)

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Number of residues/1000 residues</th>
<th>A20</th>
<th>P20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid (Asp)</td>
<td></td>
<td>117.50 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.60 ± 0.91&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Alanine (Ala)</td>
<td></td>
<td>93.13 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96.95 ± 1.59&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arginine (Arg)</td>
<td></td>
<td>44.28 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.52 ± 1.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cysteine (Cys)</td>
<td></td>
<td>4.57 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.90 ± 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Glutamic Acid (Glu)</td>
<td></td>
<td>165.59 ± 1.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>174.30 ± 2.23&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Glycine (Gly)</td>
<td></td>
<td>71.26 ± 0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.87 ± 3.72&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Histidine (His)</td>
<td></td>
<td>20.38 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.68 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Isoleucine* (Ile)</td>
<td></td>
<td>31.06 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.97 ± 1.66&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Leucine* (Leu)</td>
<td></td>
<td>84.10 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.29 ± 1.79&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Lysine* (Lys)</td>
<td></td>
<td>84.96 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.32 ± 2.86&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Methionine*(Met)</td>
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<td>33.25 ± 0.81&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Phenylalanine*(Phe)</td>
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<td>29.16 ± 2.11&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Proline (Pro)</td>
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<td>36.42 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Serine (Ser)</td>
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<td>59.93 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.19 ± 0.98&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Threonine (Thr)</td>
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<td>50.75 ± 1.16&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Tyrosine*(Tyr)</td>
<td></td>
<td>26.62 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.82 ± 0.90&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Valine*(Val)</td>
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<td>41.02 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.02 ± 0.99&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>HAA</td>
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<td>374.78 ± 1.17&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>51.98 ± 2.46&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>NCAA</td>
<td></td>
<td>283.08 ± 1.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>290.89 ± 1.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCAA</td>
<td></td>
<td>149.63 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>153.52 ± 2.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>*EAA</td>
<td></td>
<td>353.94 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>373.44 ± 4.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

A20: hydrolysate by Alcalase with a 20% DH; P20: hydrolysate by Protamex with a 20% DH; Lower case in the same row indicate significant (p < 0.05) differences among hydrolysates. AAH: hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, proline, methionine, and cysteine); AAA: aromatic amino acids (phenylalanine, tryptophan and tyrosine); AACP: positively charged amino acids (arginine, histidine, lysine); AACN: negatively charged amino acids (aspartic acid, glutamic acid); * EEA: essential amino acids (phenylalanine, valine, threonine, isoleucine, methionine, histidine, leucine and lysine).
### Table 2 – Antimicrobial activity of different hydrolysates obtained by inhibition halo obtained by the agar diffusion method

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Inhibition halo (mm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A20</td>
<td>P20</td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>6.20 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td><em>Brochothrix thermosphacta</em></td>
<td>5.57 ± 0.12&lt;sup&gt;deB&lt;/sup&gt;</td>
<td>7.67 ± 0.48&lt;sup&gt;bA&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ni</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td><em>Debaryomyces. Hanseii</em></td>
<td>10.87 ± 0.45&lt;sup&gt;bA&lt;/sup&gt;</td>
<td>7.53 ± 0.37&lt;sup&gt;bB&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Listeria innocua</em></td>
<td>5.33 ± 0.32&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ni</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em></td>
<td>ni</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>14.52 ± 0.18&lt;sup&gt;AA&lt;/sup&gt;</td>
<td>9.03 ± 0.40&lt;sup&gt;AB&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>ni</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>6.20 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ni</td>
<td></td>
</tr>
</tbody>
</table>

A20: Hydrolysate by Alcalase with a 20% DH. P20: hydrolysate by Protamex with a 20% DH. ni: no inhibition. Lower case in the same column and capital letters in the same line indicate significant (p < 0.05) differences among hydrolysates.
Table 3 - Physical and mechanical properties of agar-based film incorporated with PH or CEO

<table>
<thead>
<tr>
<th>Properties</th>
<th>Control</th>
<th>PH</th>
<th>CEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness (mm)</td>
<td>0.043b ± 0.001</td>
<td>0.044b ± 0.002</td>
<td>0.061a ± 0.012</td>
</tr>
<tr>
<td>TS (MPa)</td>
<td>27.46b ± 2.78</td>
<td>19.89b ± 1.81</td>
<td>10.16c ± 1.02</td>
</tr>
<tr>
<td>EB (%)</td>
<td>22.24b ± 3.55</td>
<td>42.70a ± 1.38</td>
<td>3.93c ± 0.15</td>
</tr>
<tr>
<td>WVP (x 10^5)</td>
<td>1.40b ± 0.04</td>
<td>3.61a ± 0.11</td>
<td>3.37a ± 0.16</td>
</tr>
<tr>
<td>(g mm/ h Pa cm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubility (%)</td>
<td>21.95b ± 1.52</td>
<td>48.86a ± 2.22</td>
<td>20.86b ± 1.99</td>
</tr>
<tr>
<td>L*</td>
<td>96.03c ± 0.14</td>
<td>96.14a ± 0.13</td>
<td>96.25a ± 0.08</td>
</tr>
<tr>
<td>a*</td>
<td>-0.51b ± 0.00</td>
<td>-0.61a ± 0.02</td>
<td>-0.54b ± 0.01</td>
</tr>
<tr>
<td>b*</td>
<td>3.65b ± 0.09</td>
<td>4.21a ± 0.16</td>
<td>3.70b ± 0.15</td>
</tr>
<tr>
<td>Y (%)</td>
<td>10.23b ± 0.20</td>
<td>9.66b ± 0.20</td>
<td>12.80a ± 0.21</td>
</tr>
<tr>
<td>Transparency</td>
<td>1.57b ± 0.06</td>
<td>1.55a ± 0.06</td>
<td>4.87a ± 0.09</td>
</tr>
</tbody>
</table>

Control: Agar-based films without the incorporation of active compounds; PH: films with the incorporation of hydrolysate; CEO: agar-based films incorporated with clove essential oil; TS: tensile strength; EB: elongation at break; WVP: water vapor permeability; L*: luminosity ranges from 0 (black) to 100 (white); a*: green-red coordinate (-a*: green, +a*: red); b*: blue-yellow coordinate (-b*: blue, +b*: yellow); Y: opacity; Equal letters on the same row indicate that there is no significant difference (p > 0.05).

Fig. 1. Overall molecular weight distribution of hydrolysate by Alcalase with a 20% degree of hydrolysis (Fig. 1a) and: hydrolysate by Protamex with a 20% degree of hydrolysis. (Fig. 1b) at optical density of 214 nm.
Fig. 2. SEM micrographs of surface (a) and cross-section (b) of agar films (control films); surface (c) and cross-section (d) of agar films incorporated with protein hydrolysate (PH films); surface (e) and cross-section (f) of clove essential oil (CEO.films).
Fig. 3. pH (a) and total basic volatile nitrogen microbial (mg TBV-N/100 g) (b) in fillet covered with agar films (control films) and agar films incorporated with protein hydrolysate (PH films) and clove essential oil (CEO.films). The statistical assays are shown in supplementary material 2.

Fig. 4. Changes in weight loss of the flounder fillets in fillet covered with agar films (control films) and agar films incorporated with protein hydrolysate (PH films) and clove essential oil (CEO.films). The statistical assays are shown in supplementary material 2.
Fig. 5. Microbiological counts (log cfu/g) of Enterobacteriaceae (a), total aerobic mesophiles (b), H2S-producers microorganisms (c), Pseudomonas spp. (d) and lactic bacteria (e) for flounder fillets fillet covered with agar films (control films) and agar films incorporated with protein hydrolysate (PH films) and clove essential oil (CEO films). The statistical assays are shown in supplementary material 3.
### Supplementary material 1

#### TVB-N

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control films</td>
<td>fA</td>
</tr>
<tr>
<td>PH films</td>
<td>fA</td>
</tr>
<tr>
<td>CEO films</td>
<td>fA</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,...) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.

#### pH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control films</td>
<td>eA</td>
</tr>
<tr>
<td>PH films</td>
<td>eA</td>
</tr>
<tr>
<td>CEO films</td>
<td>eA</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,...) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.
### Supplementary material 2

**Weight loss**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control films</td>
<td>Af</td>
</tr>
<tr>
<td>PH films</td>
<td>Af</td>
</tr>
<tr>
<td>CEO films</td>
<td>Af</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,...) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.
**Supplementary material 3**

**Enterobacteriaceae**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>fA</td>
</tr>
<tr>
<td>PH films</td>
<td>fA</td>
</tr>
<tr>
<td>CEO films</td>
<td>fA</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,...) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.

**Total aerobic mesophiles**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>eA</td>
</tr>
<tr>
<td>PH films</td>
<td>eA</td>
</tr>
<tr>
<td>CEO films</td>
<td>eA</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,...) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.

**H₂S-producers microorganisms**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>fA</td>
</tr>
<tr>
<td>PH films</td>
<td>fA</td>
</tr>
<tr>
<td>CEO films</td>
<td>fA</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,...) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.
Pseudomonas spp.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control films</td>
<td>eA</td>
</tr>
<tr>
<td>PH films</td>
<td>eA</td>
</tr>
<tr>
<td>CEO films</td>
<td>eA</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,..) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.

Lactic bacteria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control films</td>
<td>dA</td>
</tr>
<tr>
<td>PH films</td>
<td>dA</td>
</tr>
<tr>
<td>CEO films</td>
<td>dA</td>
</tr>
</tbody>
</table>

Different capital letters (A,B,C,..) in the same line indicate significant differences (p < 0.05) as a function of days of storage; Different lower case (a, b, c,...) in the same column indicate significant differences (p < 0.05) as a function of treatments.