The effect of the combined use of high pressure treatment and antimicrobial edible film on the quality of salmon carpaccio

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11 **ABSTRACT**

12 Fish carpaccio is a ready-to-eat product with a very limited shelf life. In the present
13 work, the use of high pressure treatment (HP) and/or antimicrobial edible film was
14 studied in order to improve quality and stability of salmon carpaccio. In a preliminary
15 part of the work, a film composed of gelatin plus chitosan incorporating clove essential
16 oil was selected, based on its physicochemical and antimicrobial properties. Eugenol
17 and β-caryophyllene, the main volatile components of the film, migrated to salmon
18 muscle, the release being favored by HP and storage time. Concurrently, reducing
19 power of the muscle increased, resulting in prevention of lipid oxidation derived from
20 either HP or refrigerated storage. HP treatment reduced total microbial counts by 1.5 log
21 cycles from the onset of storage, whereas the film reduced it by 2 log cycles after 3
22 days. The combination of HP and edible film exerted the most intense antimicrobial
23 effect, total bacterial counts, luminescent bacteria, H_2S-producing organisms,
24 pseudomonads, enterobacteria, and lactic acid bacteria remaining constant or under
25 detection limit over the whole storage period (11 days). The combined use of HP
26 treatment and gelatin–chitosan–clove essential oil film is an effective way of improving
27 quality and stability of salmon carpaccio.

28 **Keywords**

29 Clove; gelatin; chitosan; shelf life; volatile compounds

30

1. INTRODUCTION

Fish and seafood are much-appreciated foods worldwide owing to their nutritional quality. However, the organoleptic, hygienic, and nutritional quality of fresh fish diminishes over time, mainly as a result of microbial growth. Nowadays there is a growing trend towards consumption of minimally processed products with sensory properties similar to those of raw products. Specifically, carpaccio, sushi, and other fishery products that are consumed without thermal treatment are becoming more and more popular worldwide. In these specific cases, cutting, manipulating, and packaging processes are a source of microbial contamination that limits their shelf life under refrigerated storage, sometimes to just one day. This means that they must be produced and distributed almost daily, which is an added difficulty that hinders the availability of carpaccio and also increases its price. Consequently, the search for preservation methods in order to extend its shelf life and maintain both hygienic and sensory quality is a real challenge. Commercially, the preservation method most commonly employed is the addition of antimicrobials; however, research on new preservation treatments and technologies is necessary, owing to the growing consumer demand for a reduction of synthetic preservatives. In this connection, essential oils have demonstrated their effectiveness extending fish shelf life, and recent works can be found on this topic (Huang et al., 2018; Yuan et al., 2017). However, a current trend in food preservation is the application of hurdle treatments, as “a deliberate combination of existing and novel preservation techniques in order to establish a series of preservative factors (hurdles) that any microorganisms present should not be able to overcome” (Leistner, 1992), contributing to diminish sensorial impact. Examples of the application of various hurdles to extend shelf life and improve quality of fish can be found in the literature: salting, smoking, and high pressure on dolphinfish (Montero et al., 2007); salting,

56 modified atmosphere packaging, and oregano essential oil on sea bream (Goulas and
57 Kontominas, 2007), or high pressure and functional edible films on cold-smoked
58 sardine or trout fillets (Albertos et al., 2014; Gomez-Estaca et al., 2007). However, to
59 the best of our knowledge there is scarcely any information about fish carpaccio treated
60 by combined hurdles. Thus, the objective of the present work was to extend the shelf
61 life of a highly perishable food product, salmon carpaccio, by the application of high
62 pressure treatment in combination with an antimicrobial edible film. Furthermore, the
63 physicochemical and antimicrobial properties of the edible films were evaluated in
64 relation to the matrix employed (gelatin or gelatin plus chitosan) and the addition of
65 clove essential oil.

66 **2. MATERIALS AND METHODS**

67 **2.1. Preparation of carpaccio**

68 Carpaccio was prepared from salmon (*Salmo salar*) acquired in a local supermarket.
69 Portions of approximately 10×8×5 cm were frozen at –40 °C in a blast freezer
70 (Frigoskandia laboratory freezer, Helsingborg, Sweden) and stored frozen at –20 °C for
71 2 days prior to slicing. The portions were semi-thawed and slices ~1.5 mm thick were
72 prepared with a slicing machine. The slices were vacuum packed (≈60 g/pack) into
73 flexible bags properly separated with polyethylene film or edible film to avoid contact
74 between slices.

75 **2.2. Formulation of the edible films**

76 The film-forming solutions were prepared using commercial fish gelatin, mainly from
77 catfish (Lapi Gelatin., Florence, Italy), alone or in combination with chitosan (Guinama,
78 Valencia; deacetylation degree 95%). Gelatin film-forming solutions were prepared

79 with a concentration of 8 g of gelatin/100 mL of distilled water. For gelatin–chitosan
80 film-forming solutions, 6 g of gelatin and 2 g of chitosan per 100 mL solution were
81 used. Chitosan was previously dissolved in 30 mL of 0.15 M acetic acid. Sorbitol (0.15
82 g/g gelatin or gelatin plus chitosan) plus glycerol (0.15 g/g gelatin or gelatin plus
83 chitosan) was employed as a plasticizer for all formulations. For the clove-added films,
84 food-grade clove essential oil (Eladiet, Barcelona, Spain) was added to a concentration
85 of 0.75 mL/g biopolymer (gelatin or gelatin plus chitosan), using soya lecithin as an
86 emulsifying agent (160 mg/g oil), and homogenizing with an Ultra-Turrax blender
87 (12,000 rpm, 1 minute) (T25 basic, IKA-Werke GmbH & Co. KG, Staufen, Germany).
88 All mixtures were warmed and stirred at 45 °C to obtain a good blend, the pH was
89 adjusted to 6, and the films were obtained by casting an amount of 40 mL on Perspex
90 plates (144 cm^2) and drying at 45 °C in a forced-air oven for 15 h to yield a uniform
91 thickness [200 μm ($p \leq 0.05$)] in all cases. Four different types of films were obtained: a
92 gelatin film (G); a gelatin–chitosan film (G-Ch); a gelatin clove-added film (G-C); and a
93 gelatin–chitosan clove-added film (G-Ch-C). Prior to analyses the films were
94 conditioned in desiccators for 2 d at 22 °C to 58% relative humidity.

95 **2.3. High pressure treatment**

96 A Stansted Fluid Power Iso-Lab 900 high pressure food processor (Model:
97 FPG7100:9/2C, Stansted Fluid Power Ltd., Harlow, Essex, UK) was employed.
98 Pressure was set at 250 MPa, temperature at 7 °C and the treatment time was 15 min.
99 Temperature of the immersion medium (distilled water) was regulated by a
100 thermocouple connected to a programmed temperature controller (model IA/2230 AC,
101 INMASA, Barcelona, Spain). Pressure was increased by 2.5 MPa/s, and after high
102 pressure treatment was completed the pressure returned to that of the atmosphere after
103 approximately 3 s.

104 Four batches, all of them vacuum packed in flexible bags (type BB4L, Cryovac,
105 Barcelona, Spain), were prepared: salmon carpaccio held at atmospheric pressure
106 without active edible film (S batch), pressurized salmon carpaccio (S-HP), salmon
107 carpaccio covered with G-Ch-C edible film (S-F), and salmon carpaccio covered with
108 G-Ch-C edible film and pressurized (S-HP-F). The G-Ch-C edible film was selected
109 from the four films tested because of its physicochemical and antimicrobial properties.
110 After high pressure treatment, all batches were stored at 5 °C ± 1 °C and periodic
111 analyses were performed.

112 **2.4. Physicochemical and antimicrobial properties of the films**

113 **2.4.1. Mechanical properties**

114 A puncture test was performed in quintuplicate to determine the breaking force and
115 deformation of the films at the breaking point. Films were placed in a cell 5.6 cm in
116 diameter and perforated to the breaking point using an Instron model 4501 Universal
117 Testing Machine (Instron Co., Canton, MA, USA) with a round-ended stainless-steel
118 plunger (∅ = 3 mm) at a cross-head speed of 60 mm/min and with a 100-N load cell.
119 Breaking force was expressed in N and breaking deformation in %, according to
120 Gomez-Estaca et al. (2011).

121 **2.4.2. Water solubility**

122 It was determined as previously described by Gomez-Estaca et al. (2011). Briefly, film
123 portions measuring 4 cm^2 were placed in aluminum capsules with 15 mL of distilled
124 water and shaken gently at 22 °C for 15 h. The solution was then filtered through
125 Whatman No. 1 filter paper to recover the remaining undissolved film, which was
126 desiccated at 105 °C for 24 h. Film solubility was expressed in %.

127 **2.4.3. Antimicrobial properties**

128 The antimicrobial activity of the films was determined against the following
129 microorganisms, which were obtained from the Spanish type culture collection (CECT):
130 *Staphylococcus aureus* CECT 240, *Clostridium perfringens* CECT 486, *Listeria*
131 *monocytogenes* CECT 4032, *Photobacterium phosphoreum* CECT 4192, *Pseudomonas*
132 *aeruginosa* CECT 110, *Brochothrix thermosphacta* CECT 847, *Aeromonas hydrophila*
133 CECT 839T, *Citrobacter freundii* CECT 401, and *Shewanella putrefaciens* CECT
134 5346T. For this purpose, spread plates of BHI Agar (plus 1% NaCl in the case of *P.*
135 *phosphoreum*) were inoculated with 100 μL of these bacteria grown overnight (~10^8
136 cfu/mL), and circular pieces of the various films (1.5 cm diameter) were laid on the
137 inoculated plate's surface and after incubation the observed inhibition zones –
138 surrounding clear areas – were considered as a measurement of the antimicrobial
139 activity. The organisms were incubated at 37 °C excepting *A. hydrophila* and *S.*
140 *putrefaciens*, incubated at 30 °C, *B. thermosphacta* at 25 °C, and *P. phosphoreum* at 15
141 °C. In addition, *C. perfringens* was grown under anaerobic conditions (Gas-Pack,
142 Anaerogen; Oxoid). The inhibition area was measured using specific software for digital
143 image analysis (MIP 4 ADV, ver. 1. Consulting de Imagen Digital, S.L. & Microm,
144 Spain). Results are expressed as the percentage of inhibition with respect to the total
145 plate surface.

146 **2.5. Storage trial on salmon carpaccio**

147 **2.5.1 Microbiological analyses**

148 A total amount of 10 g of muscle was collected and placed in a sterile plastic bag
149 (Sterilin, Stone, Staffordshire, UK) with 90 mL of buffered 0.1% peptone water (Oxoid,
150 Basingstoke, UK) in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid,

151 Spain). After 1 min in a Stomacher blender (model Colworth 400, Seward, London,
152 UK), appropriate dilutions were prepared for the following microorganism
153 determinations: (i) total bacterial counts (TBC) on spread plates of Iron Agar 1% NaCl
154 incubated at 15 °C for 3 days; (ii) H_2S-producing organisms, as black colonies, on pour
155 plates of Iron Agar incubated at 15 °C for 3 days; (iii) luminescent bacteria on spread
156 plates of Iron Agar 1% NaCl incubated at 15 °C for 5 days; (iv) pseudomonads on
157 spread plates of Pseudomonas Agar Base (Oxoid) with added CFC (Cetrimide,
158 Fucidine, Cephalosporine) supplement for *Pseudomonas* spp. (Oxoid) incubated at
159 25 °C for 48 h; (v) Enterobacteriaceae on double-layered plates of Violet Red Bile
160 Glucose agar (VRBG, Oxoid) incubated at 30 °C for 48 h [after first adding 5 mL of
161 Tryptone Soy Agar (Merck, Darmstadt, Germany) and incubating at room temperature
162 for 1 h]; (vi) lactic acid bacteria (LAB) on double-layered plates of MRS Agar (Oxoid)
163 incubated at 30 °C for 72 h. All microbiological counts are expressed as the log of the
164 colony-forming units per gram (log cfu/g) of sample. All analyses were performed in
165 triplicate.

166 **2.5.2 Chemical analyses**

167 The total volatile basic nitrogen (TVBN) was determined according to the method
168 described by Antonacopoulos and Vyncke (1989) and the results were expressed as mg
169 of N per 100 g of muscle. Lipid extraction and subsequent free fatty acids determination
170 was performed as previously described by Gomez-Estaca et al. (2007), and the results
171 were expressed as percentage of free oleic acid. The thiobarbituric acid reactive
172 substances (TBARS) were determined as described in a previous work (Gomez-Estaca
173 et al., 2007) and results expressed as mg of malonaldehyde/kg muscle, based on a
174 standard constructed with 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., St. Louis,
175 MO, USA). All determinations were performed in triplicate.

2.5.3. Headspace volatiles

The analysis of the volatiles present in the salmon carpaccio and film headspaces was performed by SPME/GC/MS. Six grams of salmon slices or six grams of G-Ch-C film were accurately weighed and placed in a 20 mL glass vial that was hermetically sealed and maintained at 30 °C for 75 min prior to analysis. A PDMS-DVB-CAR fiber (Supelco Bellefonte, PA, USA) was exposed for 15 min to the headspace, then desorbed at 240 °C for 2 min into an Agilent 6890 gas chromatograph fitted to a 5973MSD Agilent mass spectrometer, operated in the positive electron ionization mode and with quadrupole mass filter. Chromatographic separations were performed on a DB-WAXetr polyethylene glycol capillary column (60 m × 0.321 mm × 0.25 μm). The oven temperature program was from 40 °C (held for 3 min) to 80 °C at 4 °C/min. Then from 80 °C to 100 °C at 8 °C/min and held for 5 min. Finally from 100 °C to 240 °C at 10 °C/min and held for 5 min. The constant flow rate of the carrier gas (99.995% helium) was 1.5 mL/min. Chromatograms and spectra were analyzed using the Chemstation software (Agilent Technologies) and compounds tentatively identified by comparison with characteristic mass spectra from the NIST08 and Wiley 7th edition mass spectral libraries (Wiley & Sons, NY). In some cases, standards were also employed for identification. Results were expressed as area counts. Furthermore, the relative composition of the film headspace was calculated and expressed in %.

2.6. Statistical analysis

Statistical tests were performed using the SPSS® computer program (SPSS Statistical Software, Inc., Chicago, Ill.). In both experiments, two-way ANOVAs for completely randomized design with a 2 x 2 factorial arrangement of treatments, were carried out. In the first experiment, the model included the fixed effects of chitosan, clove oil and their

200 interactions; and HP, edible film and its interaction effects, for the second experiment.
201 The Tukey-b test was used to the mean comparison. All significances were considered
202 to $p \leqslant 0.05$ level.

203 **3. RESULTS AND DISCUSSION**

204 **3.1. Physicochemical and antimicrobial properties of edible films**

205 Mechanical properties of the films developed are shown in Table 1. The two factors
206 studied (presence of chitosan and clove EO), as well as the interaction between them,
207 had a significant effect on both puncture force and deformation ($p \leq 0.05$). It can be seen
208 that the gelatin–chitosan compound film showed higher puncture force but lower
209 puncture deformation than the gelatin one ($p \leq 0.05$). This can be explained by the fact
210 that gelatin and chitosan interact via electrostatic and hydrogen bonding (Taravel and
211 Domard, 1995), and films made from a blend of these two polymers show higher
212 strength and lower extensibility than those made only from gelatin (Fakhreddin
213 Hosseini et al., 2013). Furthermore, this effect has been found to be especially evident
214 for gelatins of marine origin, owing to the weaker protein–protein interactions, as
215 compared to gelatins from mammals (Gomez-Estaca et al., 2011). The inclusion of
216 clove essential oil caused a marked reduction in the puncture force of both matrices
217 ($p \leq 0.05$), which was accompanied by an increase in puncture deformation that was only
218 significant ($p \leq 0.05$) for the gelatin film. From the results of mechanical properties it
219 seems clear that clove essential oil had a plasticizing effect, which agrees with a
220 previous work by Giménez et al. (2012), who worked with a gelatin–egg white film
221 incorporating the same essential oil. In the work cited the authors demonstrated the
222 interaction via hydrogen bonds of clove essential oil and protein, as well as strong
223 interference in the degree of polymer–glycerol interactions. Many studies have reported

224 a decrease in tensile strength as lipid concentration increases in protein and
225 carbohydrate matrices (Bertan et al., 2005; Yang and Paulson, 2000), owing to the
226 replacement of polymer–polymer interactions by lipid–polymer interactions.
227 Furthermore, Bertan et al. (2005) reported an increase in elongation at break resulting
228 from the addition of hydrophobic compounds to composite films based on gelatin,
229 caused by their plasticizing effect. In the present work, despite the differences, all the
230 films were malleable and easy to handle for use as slice separators.

231 Water solubility is a very important property of edible films, as it affects both the
232 integrity of the film and the release of active components. Values differed depending on
233 the film matrix (Table 1). Both chitosan and clove essential oil had a significant effect
234 on this property ($p \leq 0.05$), causing a reduction. However, the interaction between both
235 factors was not significant effect ($p > 0.05$). The effect of chitosan is consistent with the
236 establishment of the above-mentioned interactions between the two polymers, whereas
237 the effect of clove EO must be attributed to the establishment of protein–polyphenol
238 interactions, resulting in relative cross-linking of the film matrix. In this connection,
239 gelatin is generally agreed to be more prone to interact with polyphenols than globular
240 proteins because of its intrinsic open structure (Frazier et al., 2003). Furthermore, fish
241 gelatins (tuna skin) have been reported to be more reactive to polyphenols than gelatin
242 from mammals (bovine hide), owing to the lower extent of the protein–protein
243 interactions derived from the lower imino acid content (Gomez-Estaca et al., 2009).
244 This would explain the fact that the incorporation of clove essential oil in the fish skin
245 gelatin matrix in the present work reduced the water solubility, whereas a film similar in
246 composition formulated with bovine hide gelatin showed increased film solubility
247 (Gómez-Estaca et al., 2010).

Results of antimicrobial activity of the films are shown in Table 2. G film did not show any antimicrobial activity against any of the microorganisms tested. For G-Ch films the microbial inhibition was mainly limited to the film–agar medium surface, observable for *A. hydrophila*, *P. phosphoreum*, and *S. putrefaciens* (results not shown). Gelatin–chitosan interactions probably reduced active chitosan chain diffusion, limiting antimicrobial activity (Arancibia et al., 2015). However, all the microorganisms tested were sensitive to the films that included clove essential oil. In general, the least sensitive microorganisms were *C. perfringens* and *P. aeruginosa* (Table 2). Giménez et al. (2012) reported that the activity of clove essential oil was maintained in gelatin-egg white films, although both, *P. fluorescens* and *P. aeruginosa*, showed lower inhibition halo than other microbial strains such as molds and yeasts. In this regard, some molds (*Aspergillus niger* and *Penicillium expansum*) and the Gram-negative *V parahaemolyticus* were strongly inhibited by soy protein films incorporated clove (Echeverria et al., 2016). In the present work, *P. phosphoreum* and *S. putrefaciens* were significantly ($p \leq 0.05$) more sensitive to G-C film than the others, whereas *P. phosphoreum, S. putrefaciens* and *A. hydrophila* where the most sensitive ones to G-Ch-C film ($p \leq 0.05$). It is worth noting the antimicrobial effect against these microorganisms, as they are specifically involved in fish spoilage, in view of the intended application of the films developed, i.e. salmon carpaccio. Several studies reported the sensitivity of *S. putrefaciens* and *P. phosphoreum* to clove, both as essential oil or added to different edible film matrices such as gelatin, sunflower protein concentrate, gelatin-egg white or soy protein (Echeverria et al., 2016; Giménez et al., 2012; Gómez-Estaca et al., 2010; Salgado et al., 2013). In addition, the antimicrobial activity against *L. monocytogenes* is also very interesting for the intended application. This is because carpaccio is a ready-to-eat product that is consumed without previous

thermal treatment, while *L. monocytogenes* is a ubiquitous pathogenic microorganism that has the ability to grow at refrigeration temperature (Montero et al., 2007). Consequently, the search for anti-listerial treatments that limit the growth of this important pathogen is necessary and interesting. With regard to the effect of film matrix on antimicrobial activity against the various microorganisms, no clear effect was observed, as some microorganisms were more sensitive to the G-C film, some to the G-Ch-C film, and some did not show significant differences.

3.2. Shelf life of salmon carpaccio

The initial microbial count of the salmon was 4.2 log cfu/g. After a lag period (3 days) the control batch resumed growth, reaching 6.40 log cfu/g at the end of the period studied. The gelatin–chitosan film with clove showed hardly any effect during the first days ($p>0.05$), but a significant ($p\leq0.05$) reduction in the S-F batch was observed from day 3, producing 2 log cycles of difference from the control salmon, which was maintained until almost the end of storage (Figure 1). This behavior has already been reported in previous works (Gómez-Estaca, López De Lacey, et al., 2009; Gómez-Estaca et al., 2010). In the present work, high pressure treatment (250 MPa/15 min) reduced the initial microbial load of the salmon by about 1.5 log cycles ($p\leqslant0.05$). After a significant ($p\leq0.05$) increase in counts at day 3 of storage, the S-HP batch evolved similarly to the control, but maintaining this initial difference, showing significant differences ($p\leq0.05$). Ojagh et al. (2011) reported a reduction (5 log units) in salmon counts due to pressure (300 MPa/5 °C/10 min). The effect of the combined treatment (HP plus active film) on total viable bacteria was less effective than that for the single HP treatment ($p\leq0.05$) (Figure 1), with an initial reduction in total viable bacteria of 0.5

296 log. However, unlike the other batches, counts in S-F-HP remained constant during the
297 whole storage period ($p>0.05$).

298 The H_2S-producing microorganisms initially registered values close to 4 log cfu/g in the
299 S batch (Figure 1). These microorganisms are considered as indicators of presumptive
300 *S. putrefaciens* (Gómez-Estaca et al., 2010). After an adaptation period of more than a
301 week in which no growth was observed ($p>0.05$), these microorganisms resumed
302 growth ($p\leq0.05$) and reached values close to 5.8 log cfu/g. This group remained below
303 the limit of detection in the other batches with the exception of S-F, which rose at the
304 end of storage. On the other hand, the evolution of pseudomonads was similar to that of
305 total microorganisms, and this group was considered as the major form of microbiota in
306 the control salmon. It is worth noting that HP treatment caused a significant inhibition at
307 day 0 ($p\leq0.05$), but counts remained constant in ≈2 log cfu/g from day 3 without further
308 exponential growth ($p>0.05$). In the case of lactic acid bacteria, high pressure did not
309 reduce counts at day 0 ($p\leq0.05$), showing the resistance of this group to the pressure
310 treatment (Figure 1). In spite of this, as storage went on microbial counts of pressurized
311 batches generally remained below those of untreated batch ($p\leq0.05$). A similar trend
312 was observed for the batches covered with the active film, showing counts below those
313 of untreated batch ($p\leq0.05$). Presumptive *P. phosphoreum*, as counts of luminescent
314 colonies (Gómez-Estaca et al., 2010), was only detected in the control salmon at the
315 final stages. The sensitivity of *P. phosphoreum* to clove in a model system, added in
316 sunflower protein concentrate films (Salgado et al., 2013) and in gelatin–chitosan films
317 (Gómez-Estaca et al., 2010), has been reported previously. Enterobacteria were found
318 initially, probably owing to contamination during handling of the salmon. However,
319 pressure treatment inhibited their growth ($p\leq0.05$), once again revealing the ability of
320 HP treatment to preserve fish quality and its suitability as a post-packaging treatment

(Figure 1). Regarding the effect of active film, S-F batch showed a similar microbial load to untreated batch at day 0 ($p>0.05$), but from day 3 of storage onwards, they were not detected anymore.

High pressure exerted a significant effect ($p\leq0.05$) over all the microorganisms tested, whereas active film significantly affected total viable bacteria, H_2S-producing microorganisms and enterobacteriaceae ($p\leq0.05$). This means that *Pseudomonas* spp. and lactic acid bacteria were not significantly affected by the presence of film ($p>0.05$). However, the interaction between film and storage time did have an effect over all the microbial groups ($p\leq0.05$), indicating that the active film reduced microbial growth during storage. The same was also true for the interaction between HP and storage time ($p\leq0.05$). Regarding the interaction between HP treatment and active film, a significant effect was observed for all microorganisms ($p\leq0.05$), indicating an improvement of the antimicrobial effect thanks to the combined treatment.

Considering the evolution of these microbial groups, it seems that, although pseudomonads were the majority flora in the control batch, lactic bacteria seemed to predominate in the other batches for several reasons: LAB were less affected by pressure (as previously mentioned), by the presence of chitosan in the film formulation that might lead to slightly acidic conditions (Lopez-Caballero et al., 2005), and perhaps by a slight limitation of oxygen on the surface of the fish that could favor growth of this group.

The present work deals with obtaining a minimally processed seafood product in which, apart from vacuum packing and refrigerated storage, 2 barriers are identified: high pressure and active edible film (whose main antimicrobial components are chitosan and clove essential oil). Various mechanisms have been proposed for the inactivation of

345 microorganisms by these barriers. Effects of high pressure treatment that have been
346 described include modifications of the nucleus and of intracellular organelles, with
347 compression of gas vacuoles, cell lengthening, separation of the cell membrane from the
348 cell wall and the formation of pores, modifications of the cytoskeleton and strand
349 formation, coagulation of cytoplasmic protein, release of intracellular constituents, etc.
350 (Shimada et al., 1993). DNA replication and transcription and protein synthesis may be
351 affected, as well as several microbial enzymatic systems, the cell membrane being a
352 major target for pressure inactivation (Cheftel, 1995).

353 Terpenes seem to be responsible for the activity of essential oils; the hydrophobic nature
354 of their components leads to the partition of lipids in the cell and mitochondria
355 membrane, enhancing permeability, which is associated with loss of ions and reduction
356 of membrane potential, collapse of proton pump, and depletion of ATP (Sikkema et al.,
357 1994; Turina et al., 2006). The outer membrane around the cell wall makes Gram-
358 negative bacteria more resistant (Ratledge and Wilkinson, 1988). However, Gómez-
359 Estaca et al. (2010) described the effectiveness of clove edible films for reducing Gram-
360 negative species (H_2S-producing microorganisms, pseudomonads, and enterobacteria)
361 in comparison with lactic acid bacteria during storage of chilled cod. On the other hand,
362 chitosan becomes a barrier to microorganisms because of its antimicrobial properties,
363 which are related to alterations in the permeability barrier of the outer membrane in
364 Gram-negative bacteria (Helander et al., 2001), and although its bifidogenic effect has
365 been reported previously (Lee et al., 2002), sometimes both Gram-positive and Gram-
366 negative flora are similarly inhibited by chitosan (Arancibia et al., 2015).

367 The above-mentioned mechanisms contribute to the fact that the counts of the S-F-HP
368 batch remained constant or below the limit of detection in the 6 groups of
369 microorganisms studied. The Gram-negative flora were more susceptible to the

370 treatments applied (high pressure and chitosan), and thus the shelf life of salmon could
371 be increased. In addition, the essential oil acts mainly on Gram-positive flora and
372 inhibits *L. monocytogenes*, according to results in the model system obtained in the
373 present work. For all these reasons, the hurdles applied to salmon carpaccio make it
374 possible to obtain a stable and safe product.

375 The initial pH recorded was 6.48–6.64, depending on the treatment applied (Figure 2).
376 The pH of the S and S-HP batches remained practically constant, while in the film-
377 coated batches (S-F and S-F-HP) it tended to increase ($p \leqslant 0.05$). Results indicated that
378 only the factors active film and storage time, as well as their interaction, exerted an
379 effect on pH ($p \leq 0.05$). With initial values of 6.5 in fresh salmon, Ojagh et al. (2011)
380 reported that pH did not increase during the storage of pressurized salmon (300 MPa, 10
381 min, 5 °C or 40 °C), covered or not covered with fish gelatin–lignin film. In the present
382 work, although the presence of the essential oil raised the pH slightly, the values
383 remained $\leqslant 6.9$ throughout the storage period. This supports the idea that the
384 antimicrobial effect of the film was not due to an acidification effect.

385 TVBN, an index of basic compounds mainly produced by changes in muscle due to
386 microbial growth, is shown in Figure 3. Initially the salmon registered 10.1 mg
387 TVBN/100 g muscle. This value is lower than that found by Ojagh et al. (2011) in raw
388 fresh salmon (19.1 mg TVBN/100 g muscle) . The initial concentration increased
389 slightly ($p \leq 0.05$) with the application of the chitosan–clove film and/or high pressure
390 treatment. The volatile compounds remained more or less constant, reaching values
391 below 16 mg TVBN/100 g muscle at the end of conservation (far from the 30 mg
392 TVBN/100 g of muscle established as the limit for fresh fish). A reduction in
393 accumulation of base compounds in cod patties coated with a chitosan–gelatin blend

394 solution has been reported (Lopez-Caballero et al., 2005). It has also been reported that,
395 regardless of the presence of films (lignin–gelatin), high pressure treatment in salmon
396 (300 MPa, 10 min, 5 and 40 °C) practically does not change the TVBN values (Ojagh et
397 al., 2011). However, with an initial concentration of 12.9 mg TVBN/100 g of muscle,
398 cod covered with chitosan–clove film exceeded 35 mg TVBN at 10 days of storage at 2
399 ± 1 °C (Gómez-Estaca et al., 2010), which shows the different behavior of fish species
400 during storage. According to the microbiological results (Figure 1), the control batch
401 was the one with the highest counts but not the highest production of TVBN. In fact,
402 there was even a slight decline in TVBN in the control salmon at the end of the trial,
403 possibly owing to exudate, which was minimized in the covered batches because the
404 films were able to retain it. At the end of storage the salmon did not show signs of
405 spoilage. In spite of the microbial counts, the predominant spoiler organisms under
406 these treatment conditions were not present in sufficient numbers to accumulate
407 metabolites and to produce sensory rejection.

408 Results of lipid oxidation as measured by TBARS are shown in Figure 4. With regard to
409 the effect of processing at day 0, a clear lipid oxidation was produced in both
410 pressurized batches ($p \leq 0.05$). HP-derived lipid oxidation is thought to be due to
411 denaturation of haemoproteins and consequent release of haeminic iron, which catalyzes
412 oxidation reactions (Cheftel and Culioli, 1997), and it has previously been reported for
413 many fish species, including salmon carpaccio (de Oliveira et al., 2017; Gómez-Estaca,
414 López-Caballero, et al., 2009). The batch that presented the lowest oxidation value
415 ($p \leq 0.05$) at the beginning of storage was the S-F one, i.e. the one without HP treatment
416 covered with the active film. Indeed, a significant effect of the presence of film was
417 observed ($p \leq 0.05$). From day 5 of storage onwards, lipid oxidation of uncovered batches
418 (S and S-HP) increased significantly ($p \leq 0.05$), whereas the covered ones (S-F and S-F-

419 HP) remained stable from an oxidative point of view ($p>0.05$), attributable to the
420 antioxidant effect of the clove essential oil incorporated in the films (Giménez et al.,
421 2012). Furthermore, an effect of the interaction of active film and high pressure was
422 observed ($p\leq0.05$). In this regard, release of Folin–Ciocalteu reactive substances from
423 film to muscle was evidenced, together with a concomitant increase in muscle
424 antioxidant activity, which was more intense when active film and high pressure were
425 applied in combination (Figure 5). Taking into consideration the absolute TBARS
426 values attained, lipid oxidation can be considered low in the S-F and S-F-HP batches,
427 whereas in the S and S-HP ones the value of 1 mg MDA/kg muscle, which is considered
428 as the onset of oxidative rancidity (Giménez et al., 2005), was achieved. On closer
429 examination of Figure 5 it is evident that HP treatment prompted the release of active
430 substances from the film, as both the Folin–Ciocalteu reactive substances and the FRAP
431 activity were higher in the S-F-HP batch than in the S-F one at day 0. As storage
432 continued, the film gradually released the active compounds, reaching a maximum at
433 the end of storage which was not significantly different in the two covered batches. The
434 same trend was observed in a previous work in which gelatin films with the addition of
435 oregano aqueous extract were used to cover smoked sardine (*Sardina pilchardus*) fillets
436 and then pressurized at 300 MPa/20 °C/15 min (Gomez-Estaca et al., 2007).

437 Besides oxidation, lipids may undergo enzymatic hydrolysis resulting in the
438 accumulation of free fatty acids, which are more prone to lipid oxidation. Figure 6 plots
439 the accumulation of free fatty acids in salmon muscle. HP treatment produced an
440 increase in FFA in both pressurized batches ($p\leq0.05$), in agreement with previous
441 findings by Sequeira-Munoz et al. (2006) working with pressurized carp (*Cyprinus*
442 *carpio*) at 100–200 MPa/4 °C/15 or 20 min. As storage continued, FFA gradually
443 increased in the non-pressurized samples, especially in the control one. A previous work

444 also showed lower free fatty acid accumulation in cold-smoked sardine when covered
445 with an oregano-added gelatin film rich in phenolic compounds (Gomez-Estaca et al.,
446 2007). In contrast, FFA decreased in the pressurized batches from the maximum
447 reached at day 2 until the end of storage, probably owing to degradative oxidation, as
448 this effect was concomitant with the accumulation of TBARS.

449 A study of the volatiles present in the headspace of the salmon carpaccio was conducted
450 in order to investigate the possible accumulation of oxidation markers, as well as to
451 evaluate the migration of components from the films to muscle. The list of the main
452 compounds found in the salmon headspaces is presented in Table 3, in which
453 compounds that were also found in the film headspace are marked with an asterisk. The
454 most abundant compounds in the film headspace were β-caryophyllene and eugenol,
455 which accounted for 50% and 29.9% of the total, respectively. Considerable amounts of
456 α-caryophyllene (8.4%) were also found, whereas the other compounds found
457 represented less than 2% each. β-Caryophyllene and eugenol, which have been
458 documented as the main components in clove leaf essential oil (Jirovetz et al., 2006),
459 were also found in the film-covered salmon carpaccio samples (S-F and S-F-HP
460 samples). It is worth noting that although β-caryophyllene was the most abundant
461 compound in the film, eugenol was the one that migrated most to the salmon muscle
462 (Figures 7A and 7B). Migration from active packages is controlled by a combination of
463 mass transport processes that involve partition equilibrium of the agents at the
464 interphases and kinetic processes in food, headspace, coating, and substrate phases,
465 depending on the structure design (Gómez-Estaca et al., 2014). In the system developed
466 in the present work, migration is supposed to be favored as there is direct contact
467 between the film and the salmon. An effect of film on the content of eugenol and β-
468 caryophyllene in salmon muscle was observed ($p \leq 0.05$), whereas HP did not exert an

469 effect ($p>0.05$), as expected. The highly apolar nature of eugenol is the most feasible
470 cause for the high migration of this compound to a fatty fish such as salmon. Indeed, as
471 storage continued, the amounts of eugenol and β-caryophyllene increased significantly
472 ($p\leq0.05$), indicating progressive migration (Figure 7), that was also confirmed, as a
473 significant effect of the interaction of active film and storage time was observed
474 ($p\leq0.05$). With regard to the effect of high pressure treatment, it is evident that it caused
475 a significantly ($p\leq0.05$) higher release of the main film components to the salmon
476 muscle, which is in line with the Folin–Ciocalteu reactive substances determination
477 shown in Figure 5A and discussed previously. Indeed a significant effect ($p\leq0.05$) of the
478 interaction between HP and active film was found. Other compounds found in the film,
479 such as epoxycaryophyllene, methyl eugenol, acetic acid, acetyl eugenol, α-
480 caryophyllene, isoeugenol, caryophyllene alcohol, α-copahene, methyl-salicylate, and
481 limonene, also migrated to the salmon muscle, especially when high pressure treatment
482 was also applied (results not shown).

483 With regard to the volatile compounds resulting from lipid oxidation, it is worth
484 mentioning that the initial hexanal content of the salmon carpaccio (S batch) was low,
485 whereas the application of high pressure treatment (S-HP batch) resulted in a significant
486 increase ($p\leq0.05$) (Figure 7C). This is indicative of the development of some lipid
487 oxidation as a result of high pressure treatment, which is in line with the TBARS results
488 shown in Figure 4. The effect of HP was also statistically confirmed by MANOVA
489 multivariate analysis ($p\leq0.05$). Storage time also had an effect, as an increase of hexanal
490 content was observed in both batches ($p\leq0.05$), especially in the S-HP one. Contrary to
491 expectation, the hexanal contents in the S-F and S-F-HP batches were higher than those
492 in the S and S-HP ones, especially as storage continued. Taking into consideration that
493 hexanal was also present in the film (Table 3), the most feasible explanation for this

494 hexanal increase in the wrapped salmon batches is migration from the film. This was
495 supported by the statistical analysis performed, as a significant effect of the presence of
496 the active film on hexanal content was observed by MANOVA ($p \leq 0.05$). As other
497 compounds apart from hexanal that are typically observed during fish lipid oxidation,
498 such as 2,4-heptadienal, 1-penten-3-ol, 1-octen-3-ol, 2-ethylfuran, 2,3-pentanedione, Z-
499 4-heptenal, propanal, nonanal, or 2-octen-1-ol (Iglesias et al., 2009; Jónsdóttir et al.,
500 2008) were absent or only present as traces, lipid oxidation can be considered low in
501 general terms. The relatively low lipid oxidation deduced from the volatile profile
502 analysis is in accordance with the TBARS results and with the fact that no rancid odors
503 were detected in any batch from a sensory point of view. The presence of clove in the
504 films confered the salmon (SF and SF-HP batches) a spice smell, considered as pleasant
505 for this type of product consumed raw, which was similar in both batches regardless of
506 the HP treatment.

507 Other compounds found in the salmon headspace may be related to fish spoilage, such
508 as benzene derivatives (ethyl-methylbenzene or 1,3-di-*tert*-butylbenzene) (Borenstein
509 and Bunnell, 1966) or 6-methyl-5-heptenone, which has been related to oxidative
510 degradation of carotenoids (Leffingwell et al., 2015). Undecene and 2,2,4,6,6-
511 pentamethyl heptane are probably migrants from the plastic bags (Rivas-Cañedo et al.,
512 2009).

513 In conclusion, the hurdle barriers applied (i.e. gelatin–chitosan edible film with the
514 addition of clove essential oil and high pressure treatment) retarded growth of related
515 spoiler microorganisms (pseudomonads) or kept it below the limit of detection (e.g.
516 H_2S-producing organisms, enterobacteria), while lipid oxidation was low. The films
517 could be used as slice separators for salmon carpaccio slices and, in combination with
518 high pressure treatment, to improve their safety from contamination during production

519 or post-packaging. Thus, the combined treatment makes it possible to extend the shelf
520 life and obtain a stable and safe “fresh-like” minimally processed product.

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691

692 Table 1. Mechanical properties and water solubility of the films. G, gelatin film; G-C, gelatin
693 film with clove essential oil; G-Ch, gelatin–chitosan film; G-Ch-C, gelatin–chitosan film with
694 clove essential oil.

	G	G-C	G-Ch	G-Ch-C
Puncture force (N)	18.2±2.4b	7.7±1.0a	35.3±3.2c	7.9±1.6a
Puncture deformation (%)	17.1±2.3b	33.0±5.7c	6.6±2.6a	8.1±2.1a
Water solubility (%)	47.5±2.5b	35.3±4.3a	33.8±1.6a	29.0±2.3a

695 Results are means ± standard deviation. Different letters in the same row indicate significant differences
696 according to one-way ANOVA ($p \leq 0.05$).

697

698

699

700 Table 2. Antimicrobial properties of the films against selected microorganisms. Results are
701 expressed as the percentage of inhibition with respect to the total plate surface.

	G-C	G-Ch-C
S. aureus	8.94±0.53ab/x	9.30±0.05cd/x
C. perfringens	6.72±0.34a/x	7.94±0.72bc/y
L. monocytogenes	9.98±0.80ab/x	7.53±0.31b/y
P. phosphoreum	24.52±1.06c/x	27.53±0.12f/y
P. aeruginosa	5.1±0.14a/x	5.25±0.21a/x
B. thermosphacta	10.08±1.08ab/x	10.06±0.17d/x
A. hydrophila	18.40±0.15bc/x	15.92±0.21e/y
C. freundii	8.12±1.50a/x	8.68±0.50bcd/x
S. putrefaciens	34.05±7.95d/x	28.35±0.86f/x

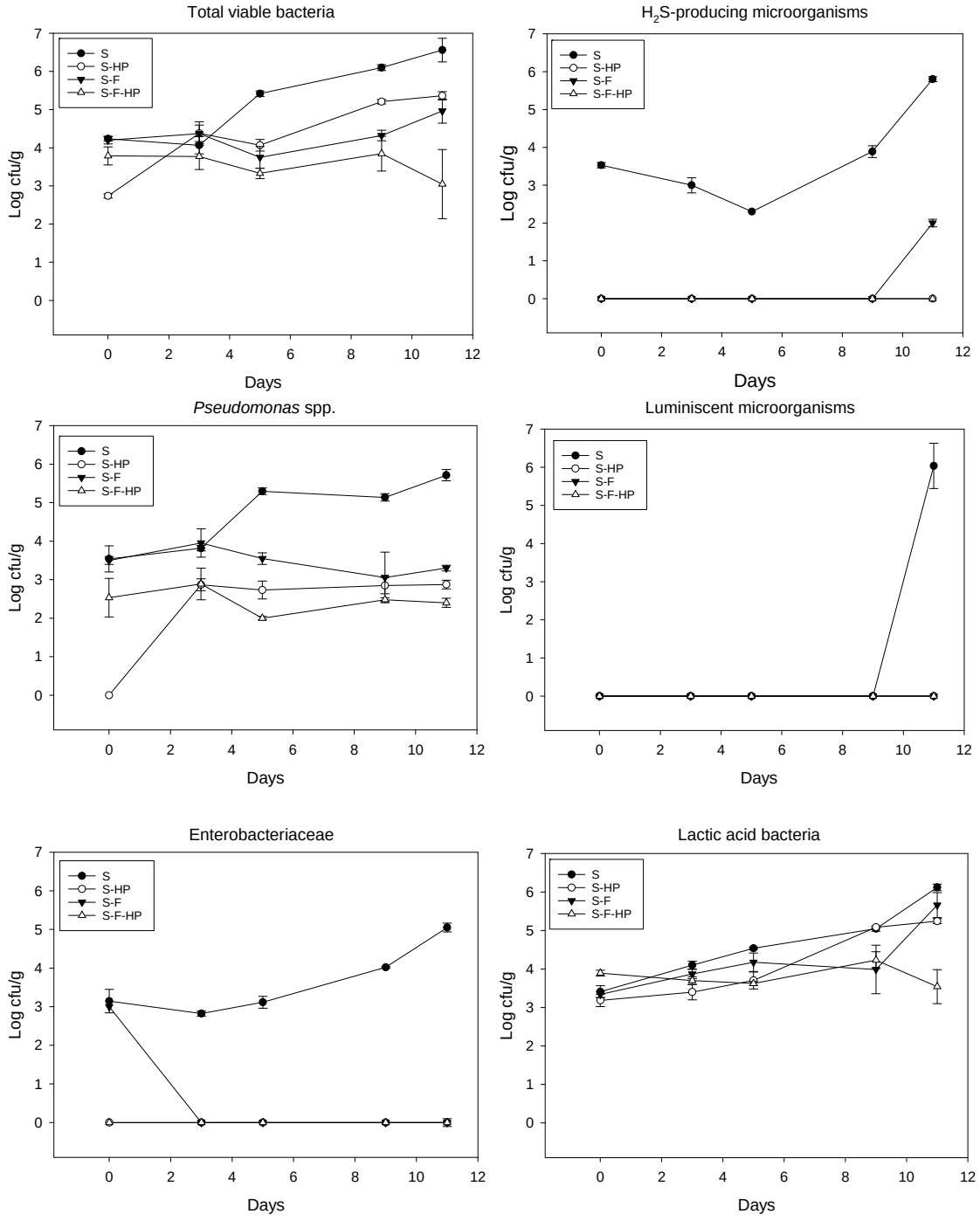
702 Different letters in the same column (a, b, c, d, e) indicate significant differences ($p \leq 0.05$) among
703 different microorganisms. Different letters in the same row indicate significant differences between the
704 two films.

705

706 Table 3. List of the main volatile compounds found in salmon carpaccio headspaces.
707 Compounds that were also found in the G-Ch-C film, which was selected for the salmon
708 carpaccio storage trial, are marked with an asterisk.

Compounds found	**Retention time**	**Identification**
Ethyl acetate	6.05	MS, Std
2,2,4,6,6-Pentamethyl-heptane	7.4	MS
2-Methyl, methyl-2-propeonate	8.9	MS
α-Pinene	9.1	MS, Std
Toluene*	9.95	MS
tert-Butyl isobutyl ether*	10.8	MS
Undecane	11.067	MS
Undecene	11.151	MS
Hexanal*	11.4	MS, Std
α-Phellandrene*	14.05	MS
Limonene*	15.3	MS, Std
3-Methyl-1-butanol	15.9	MS
Ethyl-methylbenzene	16.48	MS
Furan, 2-pentyl*	16.8	MS
3-Hydroxy-2-butanone	18.9	MS, Std
6-Methyl-5-heptenone	20.6	MS, Std
Hexanol*	21.1	MS, Std
cis-3-Hexenol	22.2	MS, Std
Nonanal	22.44	MS
1,3-di-*tert*-butylbenzene	23.4	MS
Acetic acid*	24.5	MS, Std
Furancarboxyaldehyde*	24.85	MS
α-Copahene*	25.18	MS
Benzaldehyde*	26.5	MS, Std
Linalool*	26.8	MS, Std
1-Octanol*	27.1	MS, Std
β-Caryophyllene*	28.08	MS
α-Caryophyllene*	29.7	MS
Methyl-salicylate*	32.26	MS, Std
Benzyl alcohol*	34.1	MS
Epoxycaryophyllene*	36.1	MS
Methyl eugenol*	36.32	MS
Caryophyllene alcohol*	37.02	MS
Eugenol*	38.7	MS, Std
Acetyl eugenol*	39.8	MS, Std
Unknown*	40.9	MS
Isoeugenol*	41.13	MS

709 MS: mass spectrum
710 Std: standard
711
712



713

714 Figure 1. Microbial counts during chilled storage of the various microbial groups in
715 salmon carpaccio subjected to different preservation treatments. S, control without
716 treatment; S-HP, pressurized; S-F, covered with gelatin–chitosan-clove edible film; S-F-
717 HP, covered with gelatin–chitosan–clove edible film and pressurized.
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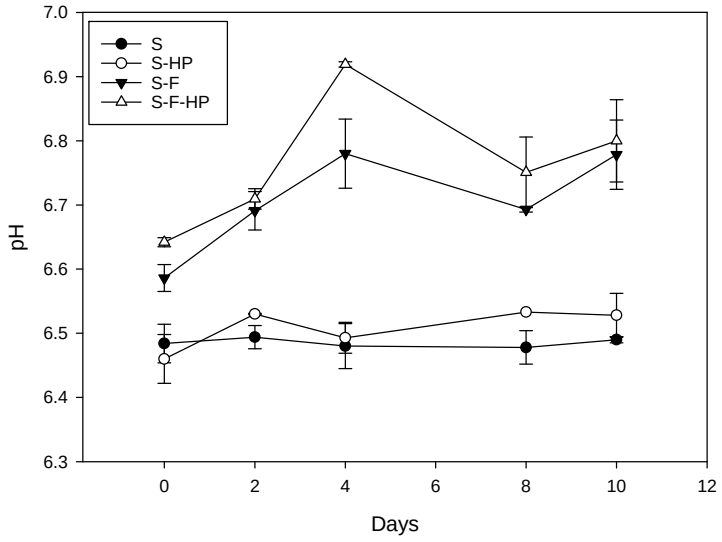

Figure 2. pH during chilled storage of salmon carpaccio subjected to different preservation treatments. S, control without treatment; S-HP, pressurized; S-F, covered with gelatin–chitosan–clove edible film; S-F-HP, covered with gelatin–chitosan–clove edible film and pressurized

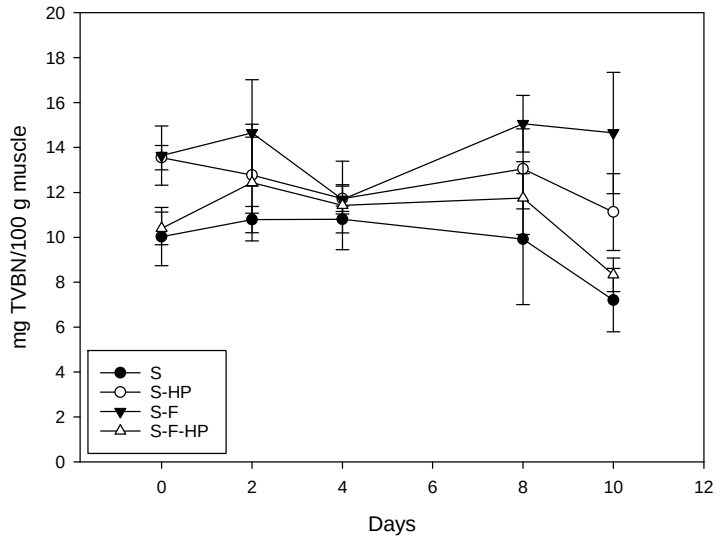

Figure 3. TVBN during chilled storage of salmon carpaccio subjected to different preservation treatments. S, control without treatment; S-HP, pressurized; S-F, covered with gelatin–chitosan–clove edible film; S-F-HP, covered with gelatin–chitosan–clove edible film and pressurized

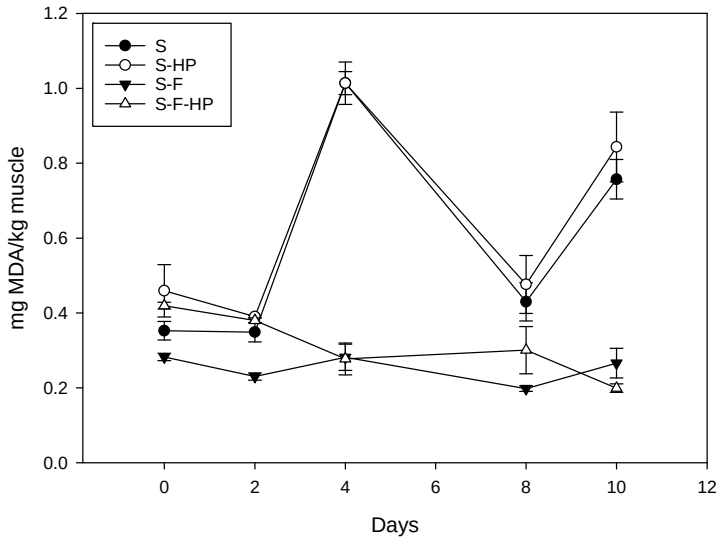

Figure 4. TBARS during chilled storage of salmon carpaccio subjected to different preservation treatments. S, control without treatment; S-HP, pressurized; S-F, covered with gelatin–chitosan–clove edible film; S-F-HP, covered with gelatin–chitosan–clove edible film and pressurized.

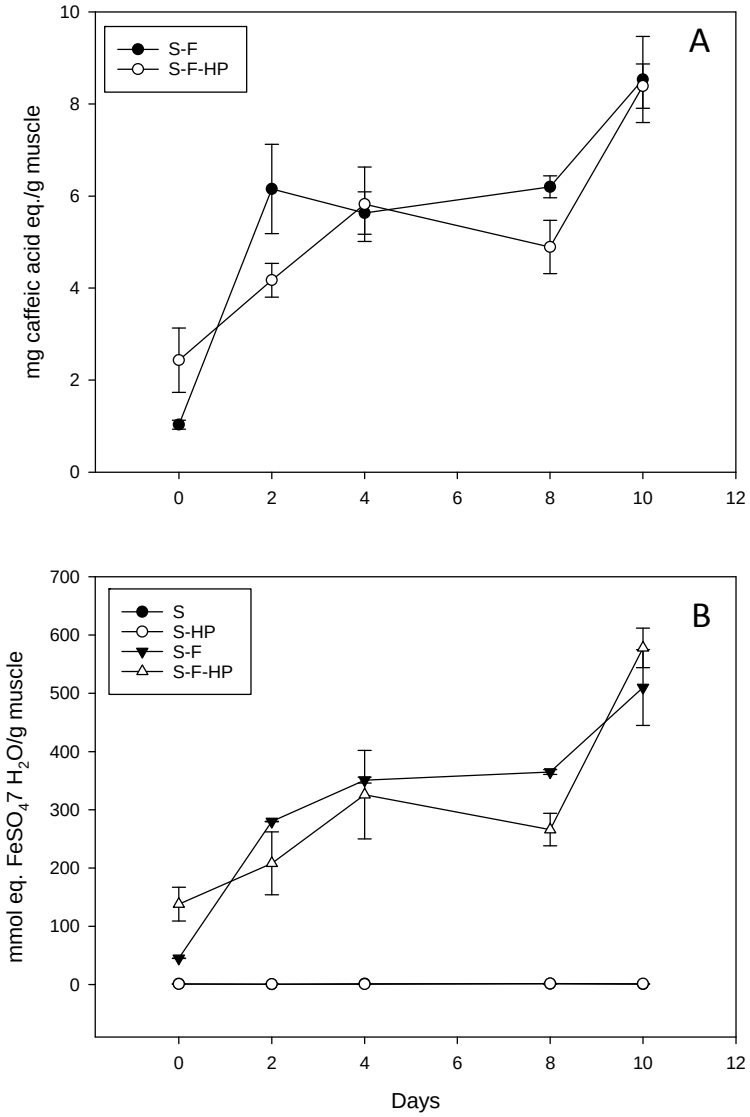

Figure 5. Folin–Ciocalteu reactive substances (A) and ferric reducing ability (B) of the various salmon batches. S, control without treatment; S-HP, pressurized; S-F, covered with gelatin–chitosan–clove edible film; S-F-HP, covered with gelatin–chitosan–clove edible film and pressurized.

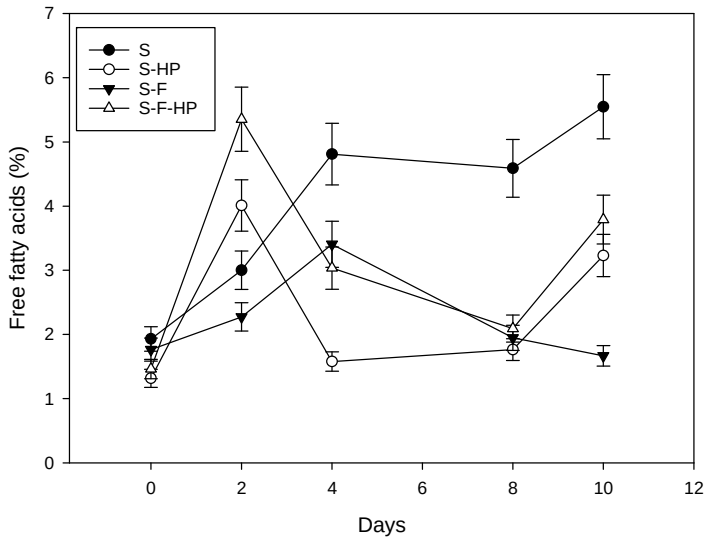

Figure 6. Free fatty acids during chilled storage of salmon carpaccio subjected to different preservation treatments. S, control without treatment; S-HP, pressurized; S-F, covered with gelatin–chitosan–clove edible film; S-F-HP, covered with gelatin–chitosan–clove edible film and pressurized.

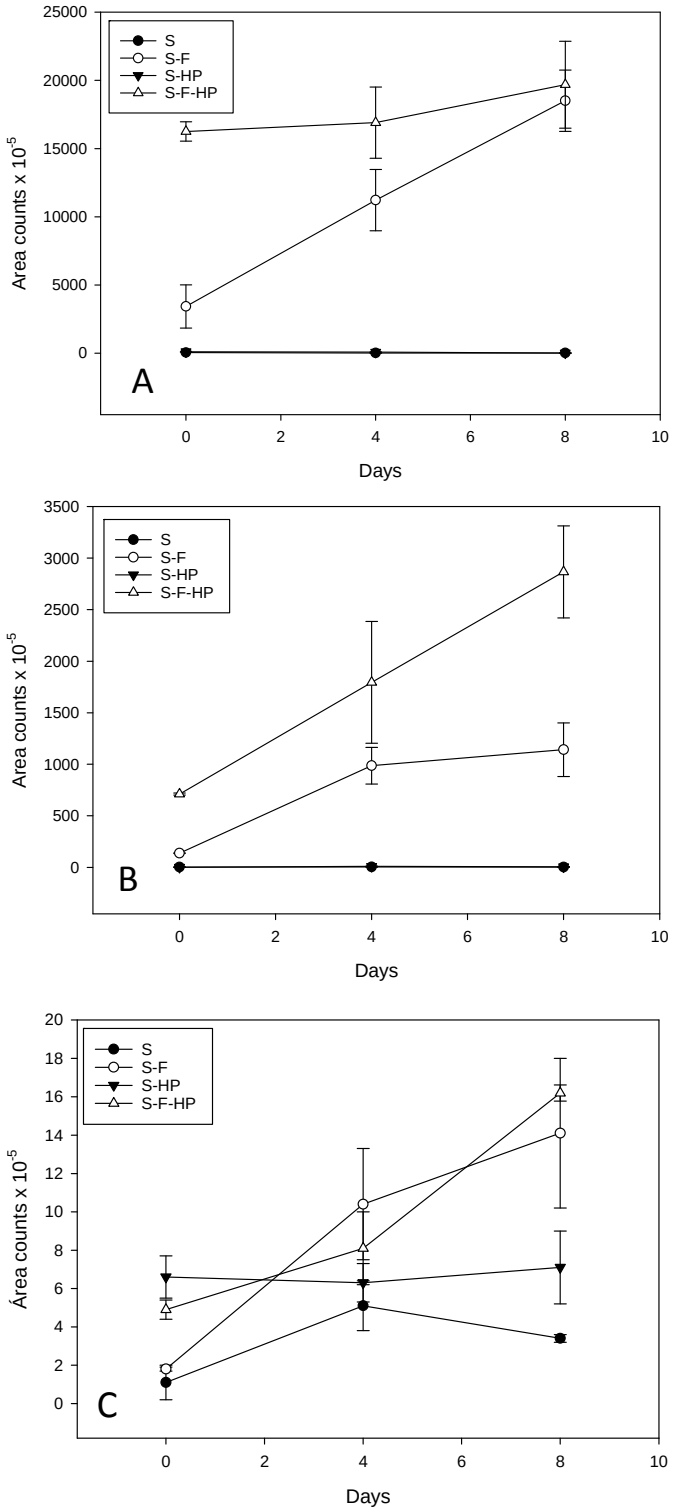

Figure 7. Eugenol (A), β-caryophyllene (B), and hexanal (C) contents in the headspace of salmon carpaccio subjected to different preservation treatments, during chilled storage. S, control without treatment; S-HP, pressurized; S-F, covered with gelatin–chitosan–clove edible film; S-F-HP, covered with gelatin–chitosan–clove edible film and pressurized.