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5 # Impact of icing systems with aqueous, ethanolic and ethanolic-
6 # aqueous extracts of alga *Fucus spiralis* on microbial and
7 # biochemical quality of chilled hake (*Merluccius merluccius*)

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SUMMARY

The potential preservative effect of an icing system including a combination of ethanolic and aqueous extracts of alga *Fucus spiralis* was analysed throughout a 13-day storage of hake (*Merluccius merluccius*). For it, flake ices including aqueous (AQ batch), ethanolic (ET batch) and ethanolic+aqueous (ET-AQ batch) extracts of this alga were separately tested. Comparison with fish kept under traditional ice proved a significant ($p<0.05$) antimicrobial effect against aerobes, psychrotrophs, proteolytic and lipolytic bacteria, derived of the presence of *F. spiralis* ethanolic extracts in the icing medium (ET and ET-AQ batches). Additionally, an inhibitory effect of both ethanol extracts was also obtained concerning lipid oxidation development (i.e., secondary and tertiary lipid oxidation compounds). However, comparison between fish corresponding to ET-AQ and ET batches did not provide differences in microbiological activity, while lipid damage assessment showed lower mean values in tertiary oxidation compound formation in hake belonging to the ET-AQ batch.

Keywords: Hake; chilling; *Fucus spiralis*; ethanolic extract; aqueous extract; antimicrobial; antioxidant.

Running Title: Combined alga extracts and hake quality.

57 **INTRODUCTION**

58 To slow down fish damage during chilled storage, a wide number of preserving
59 strategies have been combined with flake ice, these including chemical and physical
60 treatments (Campos *et al*., 2012). Among them, the inclusion in the icing medium of
61 natural preservatives such as low-molecular weight organic acids (Sanjuás-Rey *et al*.,
62 2012) and plant extracts (Quitral *et al*., 2009) has shown a remarkable inhibition of
63 microbial activity and lipid oxidation.

64 Marine algae have been reported to be part of the diet in different countries and
65 constitute a source of beneficial nutrients, such as vitamins, lipids, amino acids, trace
66 minerals and dietary fibres (Smit, 2004). Recently, brown, red and green macroalgae
67 have shown to include a wide variety of chemical constituents with potential antioxidant
68 (Halldorsdóttir *et al*., 2014) and antimicrobial (Sandsdalen *et al*., 2003) activities
69 susceptible to be applied during seafood preparation. Thus, a great number of
70 biopreservative molecules such as polyphenols, terpenes, chlorophylls, carotenoids,
71 steroids, phlorotannins, or halogenated ketones and alkanes have been isolated from
72 such algae (Serrano *et al*., 2009; Fleurence *et al*., 2012).

73 Previous studies have demonstrated that the use of different extraction methods
74 along with different single solvents or solvent mixtures (namely, water, methanol,
75 ethanol, acetone, ethyl acetate), can affect the extraction yield as well as the content on
76 phenolics and other preserving compounds (Tierney *et al*., 2013b; Babakhami *et al*.,
77 2016). In most cases, alcoholic have been the preferred solvents as being the most
78 accurate to obtain a high total phenolic content (TPC) (Wang *et al*., 2009; Farvin &
79 Jakobsen, 2013). Thus, a decreasing TPC by increasing solvent polarity has been
80 explained as a result of protein and carbohydrate precipitation in the presence of
81 alcohol, which would release some of the reversibly-bonded phenolic compounds into

82 the extracting solution (Galland-Irmouli *et al*., 1999; Burtin, 2003). However, water
83 extraction of algae has been reported to produce in most cases the highest yields
84 (Athukorala *et al*., 2003; Tierney *et al*., 2013a), this indicating that the majority of
85 water-soluble compounds such as polysaccharides, proteins and peptides would be
86 extracted and might also contribute to the overall preservative effect (Pereira *et al*.,
87 2009; Kuda & Ikemori, 2009).

88 *Fucus spiralis* is a brown alga living on the littoral shore of the Atlantic coasts of
89 Europe and North America. Olive-brown coloured, it grows about 30 cm long and is
90 generally attached to rock. Previous reports have shown its valuable nutritious content
91 (Andrade *et al*., 2013; Paiva *et al*., 2014) as well as the presence of a wide range of
92 preserving molecules (Tierney *et al*., 2013a; Peinado *et al*., 2014), this including
93 polymeric pholorotannins (Cérantola *et al*., 2006). Consequently, an inhibitory effect on
94 microbial activity and lipid oxidation of refrigerated megrim (*Lepidorhombus*
95 *whiffiagonis*) was demonstrated as a result of the inclusion of a *F. spiralis* extract in the
96 packaging film (García-Soto *et al*., 2015) and in the icing medium (Miranda *et al*.,
97 2016). In the present work, the potential preservative effect (namely, antimicrobial and
98 antioxidant behaviour) of an icing system including a combination of ethanolic and
99 aqueous extracts of *F. spiralis* was analysed throughout the chilling storage of hake
100 (*Merluccius merluccius*). For it, flake ices including aqueous, ethanolic and
101 ethanolic+aqueous extracts of this alga were prepared and separately tested as icing
102 systems.

103 **MATERIALS AND METHODS**

104 **Preparation of *F. spiralis* extracts and icing systems**

105 The lyophilised alga *F. spiralis* was kindly provided by Porto-Muiños (Cerceda, A
106 Coruña, Spain). On one hand, 15 g of lyophilised alga were mixed with absolute ethanol
107 (2 x 120 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the
108 supernatants were recovered, pulled together, diluted to 250 mL with absolute ethanol
109 and finally carried out to 6 L with distilled water. This solution was placed in
110 polyethylene bags, stored frozen at -18 ºC and later used as icing medium (ET batch).

111 At the same time, 15 g of lyophilised alga were mixed with distilled water (2 x
112 120 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the
113 supernatants were recovered, pulled together and diluted to 6 L with distilled water; in
114 order to maintain the same ethanol content in all batches, 250 mL of absolute ethanol
115 were added for the preparation of this 6-L solution. Finally, this solution was placed in
116 polyethylene bags, stored frozen at -18 ºC and later used as icing medium (AQ batch).

117 Likewise, 15 g of lyophilised alga were mixed with absolute ethanol (2 x 120
118 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the
119 supernatants were recovered, pulled together and diluted to 250 mL with absolute
120 ethanol. Additionally, the remaining lyophilised alga was mixed with distilled water (2
121 x 120 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the
122 supernatants were recovered, pooled together with the previously obtained 250-mL
123 ethanolic solution and the mixture carried out to 6 L with distilled water. This solution
124 was placed in polyethylene bags, stored frozen at -18 ºC and later used as icing medium
125 (ET-AQ batch).

126 To prepare the ice to be employed as Control batch condition, 250 mL of
127 absolute ethanol were diluted in 6 L of distilled water. The solution was packaged and

128 kept frozen in the same way as the three other ices. Before addition to fish individuals,
129 the different kinds of ice were ground to obtain ice flakes. Experimental conditions
130 (namely, content of lyophilised alga extract in the ice) employed in the present study
131 were based on previous research carried out at our laboratory concerning the
132 employment of ethanol extracts (Miranda *et al.*, 2016).

133

134 **Fish material, processing and sampling**

135 Fresh hake (102 specimens) were caught near the Galician Atlantic coast (North-
136 Western Spain), slaughtered on board and transported to the laboratory. Throughout this
137 process (about 10 h), the fish were kept in ice. The length and weight of the fish
138 individuals ranged from 27.0 to 28.5 cm and from 176 to 206 g, respectively.

139 Upon arrival to the laboratory, six individuals were separated and analysed as
140 initial fish (day 0). These individuals were divided into three groups (two specimens per
141 group) that were analysed independently in order to achieve the statistical analysis
142 (n=3). The remaining fish individuals were divided into four batches (24 individuals in
143 each batch), that were placed in different boxes and directly surrounded by different
144 kinds of ice (Control, AQ, ET and ET-AQ batches, respectively), prepared as previously
145 described. Ice was applied at a 1:1 fish:ice ratio, being all batches placed inside a
146 refrigerated (2±1 ºC) room. Boxes allowing draining of melted ice were employed for
147 hake storage. In all batches, ice was renewed in order to maintain the 1:1 fish:ice ratio.
148 Fish individuals from all batches were stored for a 13-day period, being taken for
149 analysis on days 2, 6, 9 and 13. At each sampling time, six individuals were taken from
150 each batch for analysis and divided into three groups (two specimens in each group) that
151 were studied independently (n=3).

152

153 **Microbiological analyses related to quality loss**

154 Hake samples of 10 g of white muscle were taken aseptically, mixed with 90 mL of 0.1
155 % peptone water (Merck, Darmstadt, Germany) and further homogenised in sterilised
156 stomacher bags (AES, Combourg, France) according to previous research (Ben-Gigirey
157 *et al*., 1998; Ben-Gigirey *et al*., 1999). A 0.1% peptone-water solution was employed to
158 prepare serial dilutions from the different microbial extracts.

159 Total aerobes were analysed by surface inoculation on plate count agar (PCA,
160 Oxoid Ltd., London, UK) after being incubated at 30 ºC for 48 h. Psychrotrophs
161 evolution was also investigated in PCA, being the incubation carried out at 7-8 ºC for 7
162 days. Enterobacteriaceae were analysed by pour plating using Violet Red Bile Agar
163 (VRBA) (Merck, Darmstadt, Germany) after being incubated for a 24-hperiod at 37±0.5
164 ºC. Microorganisms showing a proteolytic or lipolytic phenotype were analysed on a
165 casein-agar medium or on tributyrine-agar, respectively, after being incubated at 30 ºC
166 for 48 h, according to previous research (Ben-Gigirey *et al*., 2000).

167 For all kinds of analyses, microbial counts obtained were transformed into log
168 CFU g^{-1} muscle values before undergoing the statistical analysis. All analyses were
169 carried out in triplicate.

170

171 **Chemical analyses related to quality loss**

172 Chemical analyses related to hake quality were carried out on the white muscle.
173 Solvents and chemical reagents employed were of reagent grade in all cases (Merck,
174 Darmstadt, Germany).

175 The evolution of pH in hake muscle throughout the chilling time was determined
176 by employing a 6-mm diameter insertion electrode (Crison, Barcelona, Spain).

177 Lipids were extracted from the hake muscle by following the Bligh & Dyer
178 (1959) method. Evolution of the free fatty acid (FFA) content was determined in the
179 lipid extract of the hake muscle in agreement with the Lowry & Tinsley (1976) method,
180 which is based on a complex formation with cupric acetate-pyridine followed by
181 spectrophotometric (715 nm) determination; results are depicted as g FFA kg^{-1} lipids.
182 The peroxide value (PV) was determined spectrophotometrically (Beckman Coulter,
183 DU 640, London, UK) by peroxide reduction with ferric thiocyanate of an aliquot of the
184 lipid extract (Chapman & McKay, 1949) method; results were calculated as meq active
185 oxygen kg^{-1} lipids. The thiobarbituric acid index (TBA-i) was investigated in agreement
186 with the Vyncke (1970) procedure, based on the reaction between thiobarbituric acid
187 and an aliquot of a trichloracetic acid extract of the hake muscle; the content of the
188 resulting thiobarbituric acid reactive substances (TBARS) was spectrophotometrically
189 determined at 532 nm, calculated from a standard curve prepared with 1,1,3,3-
190 tetraethoxy-propane (TEP) and expressed as mg malondialdehyde kg^{-1} hake muscle.

191 Formation of tertiary lipid oxidation compounds was measured by fluorescence
192 spectroscopy (Fluorimeter LS 45, Perkin Elmer España; Tres Cantos, Madrid, Spain) at
193 393/463 nm and 327/415 nm in the aqueous phase resulting from the lipid extraction of
194 hake muscle (Aubourg *et al*., 1995). The relative fluorescence (RF) was calculated
195 according to: $RF = F/F_{st}$, where F is the fluorescence determined at each
196 excitation/emission wavelength pair and F_{st} is the fluorescence intensity of a quinine
197 sulphate solution (1 μg mL^{-1} in 0.05 M H_2SO_4) measured at the corresponding
198 wavelength pair. Results are expressed as the fluorescence ratio (FR), which was
199 determined as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$.

200 **Statistical analysis**

201 Data obtained from the different quality analyses (i.e., microbiologicals and chemicals)
202 were subjected to the one-way analysis of variance to explore differences resulting from
203 the effects of the icing condition; comparison of means was carried out using the least-
204 squares difference (LSD) procedure. Additionally, one-way analysis of variance was
205 also applied to study the effects of the chilling time. Analyses were carried out by
206 employing the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL,
207 USA); in all kinds of analyses, differences among batches and among chilling times
208 were considered significant for a confidence interval at the 95% level ($p<0.05$).

209 Data analyses also include correlation values among parameters (chilling time,
210 microbiological counts and chemical indices). In them, linear fittings are mentioned;
211 otherwise, the kind of fitting is expressed.

212

213 **RESULTS AND DISCUSSION**

214 **Quality evaluation by assessment of microbiological analyses**

215 The investigation of aerobic mesophiles in all four hake batches is presented in
216 Table 1. A progressive increase was observed for all fish batches as storage time
217 progressed (r^2 = 0.90-0.92). The inclusion of ethanolic *F. spiralis* extract in the icing
218 medium (ET and ET-AQ batches) provided better control of aerobes as compared to the
219 aqueous extract (AQ batch) and the Control batch. This effect was observed in a more
220 remarkable way in the ET-AQ batch at advanced storage times, where microbial
221 reduction reached a maximum of 1.66 log units on day 9 as compared with the Control
222 batch; however, higher counts were obtained in ET-AQ fish than in hake corresponding
223 to the ET batch in the 2-6-day period. Statistically-significant ($p<0.05$) differences were
224 observed between the Control batch and both ET and ET-AQ batches on days 2 and 9.

225 Remarkably, aerobe concentrations were above 6 log units in both Control and AQ
226 batches after 12 d of storage, while ET and ET-AQ levels were below such level,
227 usually considered as a limit of microbial acceptability of fish products.
228 The comparative evolution of psychrotrophs in hake batches is also shown in
229 Table 1. Psychrotrophic bacteria include members of the genera *Pseudomonas,*
230 *Moraxella, Acinetobacter, Shewanella* or *Flavobacterium*, some of them being of
231 concern for their role in fish spoilage. The evolution of psychrotrophs followed a
232 progressive increase in all four batches as storage time progressed (r^2 = 0.90-0.94). As
233 in the case of aerobes, the batches including the ethanolic extract showed lower mean
234 values than their corresponding Control and AQ batches in advanced storage times (9-
235 13-day period). Additionally, the ET-AQ extract exhibited a better control of
236 psychrotrophs growth as compared with the AQ and Control batches, these reductions
237 being significant ($p<0.05$) on day 9 and reaching a maximum of 0.91 log units. These
238 results, as those observed for the aerobes, indicate a benefit derived from the inclusion
239 of ethanolic (ET batch) or ethanolic/aqueous (ET-AQ) *F. spiralis* extracts in the icing
240 medium in terms of microbial inhibition in hake muscle. However, no differences
241 ($p>0.05$) could be concluded between ET-AQ and ET batches.
242 The evolution of Enterobacteriaceae counts (Table 1) did not reveal significant
243 ($p>0.05$) differences among all four batches. Thus, the counts of this microbial group
244 were remarkably low in all batches at all sampling times, reaching levels in the range of
245 1.00-1.43 log units. These results confirm the very good initial microbial quality of the
246 hake specimens considered in this study.
247 The investigation of microorganisms exhibiting a proteolytic phenotype is
248 presented in Figure 1. The negative effect of proteolytic bacteria in the degradation and
249 spoilage of fish muscle has been previously described (Rodríguez *et al*., 2003). The

250 results of this microbial group were quite similar as those observed for both aerobes and
251 psychrothrophs since progressive increases ($p<0.05$) were observed in all four hake
252 batches as storage time progressed (r^2 = 0.92-0.94, quadratic fitting). The presence of
253 ethanolic *F. spiralis* extracts in the icing medium (ET and ET-AQ batches) provided a
254 significant ($p<0.05$) inhibition of the growth of proteolytic bacteria on days 6 (ET
255 batch) and 13 (ET-AQ batch), as compared with the Control batch. The maximum
256 microbial inhibition was 0.82 log units on day 13 (Figure 1). As compared with the ET
257 batch, an increased protective effect by applying the ET-AQ extract was not observed
258 ($p>0.05$).

259 Figure 2 shows the comparative evolution of lipolytic bacteria in all four hake
260 batches. In agreement with the previously described microbial groups, a progressive
261 increase ($p<0.05$) was observed in the numbers of lipolytic bacteria throughout chilling
262 storage time (r^2 = 0.91-0.95). As in the case of proteolytic bacteria, the presence of
263 ethanolic extracts of *F. spiralis* (ET and ET-AQ extracts) also provided a better
264 protection of hake muscle with respect to the growth of lipolytic bacteria. Thus, lower
265 mean values were observed in the 6-13-day period, being the differences between such
266 batches and the Control batch significant ($p<0.05$) on day 13. Such differences reached
267 maximum values of 1.09 log CFU g^{-1} after 13 d of storage. As for previous microbial
268 group counts, lipolytic bacteria assessment did not lead to significant differences
269 ($p>0.05$) between ET-AQ and ET batches.

270 This study has proved a significant antimicrobial effect derived of the presence
271 of *F. spiralis* ethanolic extracts in the icing medium (ET and ET-AQ batches) on hake
272 muscle in agreement with previous reports on chilled megrim (*Lepidorhombus*
273 *whiffiagonis*) (Miranda et al., 2016) and the inclusion of lyophilised *F. spiralis* extracts
274 in a biodegradable packaging film for megrim (García-Soto *et al*., 2015). The melting of

275 the ice crystals containing *F. spiralis* extract might exert a washing effect that would
276 reduce the microbial load on the fish surface, this also limiting microbial diffusion
277 towards hake muscle.

278 Preliminary plate bioassays carried out at our laboratory showed that ethanolic
279 extracts of *F. spiralis* exhibited antimicrobial activity against *Bacillus cereus*, *Bacillus*
280 *subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas fluorescens,*
281 *Escherichia coli, Aeromonas hydrophila, Vibrio alginolyticus and Vibrio*
282 *parahaemolyticus* (data not shown). The fact that a high polyphenol content (53.3±5.0
283 GA g^{-1} lyophilised alga) was determined in ethanolic extracts of *F. spiralis* might
284 explain these results (García-Soto *et al*., 2015).

285 In most cases, alcoholic have been the preferred extraction solvents to obtain a
286 high TPC (Wang *et al*., 2009; Farvin & Jakobsen, 2013). Thus, protein, carbohydrate
287 and glycosides precipitation in alcoholic medium would easily release phenolic
288 compounds that were reversibly bounded into the extracting medium (Galland-Irmouli
289 *et al*., 1999; Burtin, 2003). Chemicals responsible for preservative activities are
290 widespread in macroalgae. The antimicrobial effect of *Fucus* spp. and other brown algae
291 (*Phaeophyceae* family) has been linked to the presence of terpenes, polyphenols
292 (Sandsdalen *et al*., 2003), oligomeric phlorotannins produced by the polycondensation
293 of phloroglucinol (1,3,5-trihydroxybenzene) units (Serrano *et al*., 2009) and
294 halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones and ketones
295 (Smit, 2004). Their inhibitory effect has been explained on the basis of their role in
296 several mechanisms, such as the inhibition of extracellular microbial enzymes,
297 deprivation of the substrates required for microbial growth, direct action on microbial
298 metabolism through inhibition of oxidative phosphorylation and complexation of metal
299 ions in bacterial growth environment (Sandsdalen *et al*., 2003; Smit, 2004).

300 Water-soluble compounds such as sulphate polysaccharides, proteins, peptides,
301 glycosides, low-molecular organic acids and salts have been reported to be present in
302 seaweed and to have potential preserving properties (Pereira *et al*., 2009; Kuda &
303 Ikemori, 2009). Thus, water extracts of *F. spiralis* have been reported to include
304 preserving phenolic acids such as chlorogenic, vanilic and caffeic acid, while such
305 compounds were not detected in ethanol extracts (Farvin & Jakobsen, 2013).
306 Additionally, previous reports have shown that water extracts of *F. spiralis* (Farvin &
307 Jakobsen, 2013) as well as of other algae (Athukorala *et al*., 2003; Wang *et al*., 2009;
308 Tierney et al., 2013a), lead to higher extraction yields as compared with other extracting
309 solvents. In spite of all these potential advantages of aqueous *F. sp*iralis extracts,
310 present results have not shown an additional preserving effect to the ethanolic extract
311 when the microbial activity is analysed.
312

313 **Quality evolution by assessment of chemical analyses**

314 Concerning the pH value in hake muscle, a progressive and significant ($p<0.05$)
315 increase was obtained in all hake batches as storage time progressed (r^2 = 0.82-0.93,
316 quadrating fitting) (Table 2). Comparison of samples corresponding to the different
317 icing conditions did not lead to significant differences ($p>0.05$). However, lower mean
318 values were observed throughout the 6-13-day period in fish stored under ET-AQ
319 condition when compared with their counterpart Control.
320 Increases in the pH value of fish muscle corresponds to the accumulation of
321 alkaline compounds, such as trimethylamine, ammonia and other nitrogen-including
322 compounds, which are mostly derived from microbial activity (Sandsdalen *et al*., 2003).
323 Thus, accurate correlation values were obtained in the present study between the pH
324 value and the different microbial parameters, especially with the lipolytic counts (r^2 =

325 0.86-0.93). Contrary to the present results, previous studies have reported an inhibition
326 of pH increase as a result of using other natural preservative compounds (i.e., oregano
327 and rosemary extracts) in ice during the chilled storage of other marine species (Quitral
328 *et al*., 2009). Also in opposition to the actual research, a previous work reported that the
329 inclusion of *F. spiralis* ethanolic extract in the icing system led to lower pH values in
330 megrim (*Lepidorhombus whiffiagonis*) muscle (Miranda *et al*., 2006).

331 A sharp and progressive FFA formation ($p<0.05$) was observed in all hake
332 batches as chilling time increased (r^2 = 0.90-0.95) (Table 2). Additionally, accurate
333 agreements between FFA and most microbial counts were also observed (r^2 = 0.86-
334 0.93). Throughout the whole study, lower mean values were obtained in samples
335 corresponding to the ET and ET-AQ batches when compared with their counterparts
336 from Control and AQ conditions. Consequently, a partial inhibitory effect on lipid
337 hydrolysis development was concluded as a result of the presence of ethanolic extracts
338 in the icing medium. However, no significant differences ($p>0.05$) could be obtained
339 between ET-AQ and ET batches. Interestingly, samples corresponding to the AQ batch
340 led to the highest mean values, being differences significant ($p<0.05$) at days 2, 9 and
341 13 when compared with fish belonging to the Control condition. An enhancement of
342 lipid hydrolysis development was also observed in chilled minced Atlantic mackerel
343 (*Scomber scombrus*) previously treated with an aqueous extract of *Polysiphonia*
344 *fucoides* (Babakhani *et al*., 2016). However, no effect was observed in the same study
345 for an aqueous extract of *Fucus serratus*.

346 Primary and secondary lipid oxidation was measured by the peroxide and TBA
347 values, respectively (Table 3). Results obtained for both parameters (lower than 6.42
348 and 1.03, respectively) can be considered low, so that a marked lipid oxidation
349 development was not reached in the actual experiment. A general increase ($p<0.05$) for

350 the PV was observed in the 0-9-day period, this being followed by a general decrease at
351 the end of the storage time. This decrease can be explained as a result of peroxides
352 breakdown, greater than their formation at such advanced storage time. Higher PV were
353 obtained in most cases in samples corresponding to ET and ET-AQ batches as
354 compared with their Control and AQ counterparts. A marked retention of peroxide
355 content is concluded as a result of the ethanolic extract presence in the icing system; this
356 retention did not provide significant differences ($p>0.05$) between ET-AQ and ET
357 batches.

358 A TBA-i increase ($p<0.05$) was obtained in all hake batches throughout storage
359 time (r^2 = 0.76-0.91). A marked increase ($p<0.05$) at day 13 was observed in most
360 samples, this being in agreement with the general peroxide drop observed. Comparison
361 among the different samples showed lower mean values in ET and ET-AQ batches than
362 in their Control and AQ counterparts. Differences were found to be significant ($p<0.05$)
363 when compared with Control batch at day 9 (ET samples) and at day 13 (ET-AQ
364 samples). As a result, a partial inhibition of secondary lipid oxidation development is
365 concluded in batches including an ethanolic extract in the icing system. However, no
366 significant differences ($p>0.05$) could be obtained between ET-AQ and ET batches.

367 A marked increase ($p<0.05$) of the FR value was obtained for all kinds of hake
368 samples at day 2 (Table 3), that was followed by a period of negligible changes in all
369 fish samples (2-9-day period). This lack of FR increase with increasing chilling times is
370 in agreement with the above-mentioned data concerning the relatively low formation of
371 primary and secondary lipid oxidation compounds. Comparison among the different
372 icing conditions showed lower mean values for the whole storage time for samples
373 corresponding to the ET-AQ batch. Such differences were found significant ($p<0.05$)
374 throughout the 2-9-day period when compared with Control samples. When compared

375 with the ET batch, fish corresponding to the ET-AQ condition showed lower mean
376 values throughout the whole storage period, these differences being significant at day 9.
377 Consequently, an inhibitory effect on tertiary lipid oxidation compounds formation is
378 concluded by employing an icing medium including both ethanolic and aqueous extracts
379 of *F. spiralis*.

380 In agreement with their photosynthetic role, algae are known to be exposed to a
381 strong combination of light and oxygen. Thus, their natural content on antioxidant
382 substances has been reported to be responsible for the lack of structural damage in their
383 organs (Smit, 2004). The antioxidant behaviour and reducing ability of ethanolic
384 extracts of *F. spiral*is were previously proved by means of in-vitro DPPH and FRAP
385 tests (Cérantola *et al*., 2006; Andrade *et al*., 2013; Peinado *et al*., 2014). This alga
386 showed a profitable content on polyphenols (90-205 µg phloroglucinol equivalents mg^{-1}
387) (Tierney *et al*., 2013a) and α-tocopherol (511.4 mg kg^{-1}) (Paiva *et al*., 2014). In a
388 structural study (Cérantola *et al*., 2006), *F. spiralis* showed the simultaneous production
389 of two types of polymeric phlorotannins of the fucol and fucophloretol classes,
390 respectively. Additionally, Tierney *et al*. (2013b) provided the identification of different
391 active compounds by quadrupole time-of-flight mass spectrometry (Q-TOF-MS), this
392 study supporting the assumption that pholorotannins present would be involved in the
393 antioxidant behaviour. Further, an inhibitory effect on lipid oxidation development was
394 demonstrated by its inclusion in a biodegradable packaging film (García-Soto *et al*.,
395 2015) and in the icing medium (Miranda *et al*., 2016) employed during megrim (*L.*
396 *whiffiagonis*) refrigeration.

397 In contrast with the results of the microbiological analyses, some differences
398 concerning chemical quality between ET and ET-AQ batches could be observed in the
399 present study. Thus, a lower tertiary lipid oxidation compounds formation could be

depicted for ET-AQ samples. This difference could be explained as a result of increasing the presence of relatively polar compounds (namely, sulphate polysaccharide, proteins, peptides, glycosides, low-molecular organic acids and salts), which have been reported to participate in the lipid oxidation inhibition (Galland-Irmouli *et al*., 1999; Burtin, 2003; Fleurence *et al*., 2012; Peinado *et al*., 2014). Thus, water extracts of *F. spiralis* have been reported to include antioxidant phenolic acids such as chlorogenic, vanilic and caffeic acid, while such compounds were not present in the ethanolic extract (Farvin & Jakobsen, 2013). Additionally, previous studies have shown that water extracts of different algae, these including *F. spiralis*, led to higher yields than extracts prepared with other solvent type (Athukorala *et al*., 2003; Tierney *et al*., 2013a; Farvin & Jakobsen, 2013).

CONCLUSIONS

This study proved a significant ($p<0.05$) antimicrobial (aerobes, psychrotrophs, proteolytic and lipolytic bacteria) effect derived of the presence of *F. spiralis* ethanolic extracts in the icing medium (ET and ET-AQ batches) of hake muscle. However, no significant differences could be observed between ET-AQ and ET batches. Concerning lipid damage (hydrolysis and oxidation) development, an inhibitory effect of both kinds of ethanolic extracts (ET-AQ and ET batches) was also observed (namely, secondary and tertiary lipid oxidation compounds assessment), this effect being greater in the case of fish belonging to the ET-AQ batch (i.e., tertiary lipid oxidation). This higher preservative effect derived of the use of an icing medium including both ethanolic and water alga extracts is explained on the basis of the presence of a wider range of preserving molecules (namely, lipophilic and water-soluble).

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547 **FIGURE LEGENDS**

548

549 **Figure 1**: Proteolytic bacteria assessment* in chilled hake muscle stored under different

550 icing conditions**.

551 * Mean values of three replicates (n = 3); standard deviations are indicated by bars. For

552 each chilling time, mean values accompanied by different letters (a, b) indicate

553 significant ($p<0.05$) differences as a result of the icing condition.

554 ** Abbreviations of icing conditions as expressed in Table 1.

555

556 **Figure 2**: Lipolytic bacteria assessment* in chilled hake muscle stored under different

557 icing conditions**.

558 * Mean values of three replicates (n = 3); standard deviations are indicated by bars. For

559 each chilling time, mean values accompanied by different letters (a, b) indicate

560 significant ($p<0.05$) differences as a result of the icing condition.

561 ** Abbreviations of icing conditions as expressed in Table 1.

TABLE 1

Aerobes, psychrotrophs and Enterobacteriaceae count assessment (log CFU g^{-1} muscle)* in hake muscle stored under different icing conditions**

Chilling time (days)	**Aerobes counts**				**Psychrotrophs counts**				**Enterobacteriaceae counts**			
	Control	AQ	ET	ET-AQ	Control	AQ	ET	ET-AQ	Control	AQ	ET	ET-AQ
0	2.10 (0.17)				2.10 (0.17)				1.00 (0.00)			
2	3.61 c (0.12)	3.09 ab (0.14)	2.64 a (0.30)	3.17 b (0.17)	3.13 a (0.58)	3.10 a (0.16)	3.11 a (0.46)	3.25 a (0.31)	1.00 a (0.00)	1.00 a (0.00)	1.00 a (0.00)	1.00 a (0.00)
6	4.10 b (0.36)	3.36 ab (0.66)	3.38 a (0.19)	3.99 b (0.19)	3.95 a (0.53)	3.14 a (0.70)	3.75 a (1.00)	4.07 a (0.64)	1.16 a (0.28)	1.00 a (0.00)	1.00 a (0.00)	1.36 a (0.32)
9	5.82 c (0.20)	5.54 bc (0.33)	5.15 b (0.40)	4.16 a (0.41)	6.42 b (0.19)	6.53 b (0.50)	6.06 ab (0.49)	5.51 a (0.15)	1.10 a (0.17)	1.43 a (0.57)	1.23 a (0.40)	1.00 a (0.00)
13	6.05 ab (0.56)	6.52 b (0.42)	5.37 a (0.22)	5.15 a (0.51)	7.21 ab (0.11)	7.98 b (0.17)	6.34 a (0.96)	6.35 ab (1.59)	1.23 a (0.24)	1.32 a (0.55)	1.00 a (0.00)	1.00 a (0.00)

* Mean values of three replicates (n=3); standard deviations are indicated in brackets. Mean values followed by different letters indicate significant differences ($p<0.05$) as a result of the icing condition.

** AQ, ET, and ET-AQ abbreviations denote icing conditions including aqueous, ethanolic and ethanolic+aqueous alga extracts, respectively, as expressed in the Material and Methods section. Control batch corresponds to traditional ice prepared without any alga extract.

TABLE 2

Evolution of pH value and free fatty acid content (g kg^{-1} lipids)* in hake muscle stored under different icing conditions**

Chilling time (days)	**pH value**				**Free fatty acids content**			
	Control	AQ	ET	ET-AQ	Control	AQ	ET	ET-AQ
0	6.46 (0.05)				16.2 (2.7)			
2	6.54 a (0.08)	6.56 a (0.07)	6.46 a (0.16)	6.66 a (0.05)	21.2 b (0.8)	30.9 c (1.4)	17.8 ab (2.3)	17.2 a (1.1)
6	6.67 a (0.13)	6.58 a (0.10)	6.69 a (0.10)	6.60 a (0.11)	25.0 ab (2.8)	33.6 b (6.6)	23.7 ab (2.8)	23.2 a (1.5)
9	6.67 a (0.05)	6.75 a (0.16)	6.76 a (0.10)	6.61 a (0.07)	26.2 a (2.0)	55.4 b (3.3)	25.7 a (1.9)	25.1 a (2.6)
13	7.06 a (0.11)	6.96 a (0.02)	6.93 a (0.05)	6.92 a (0.16)	39.6 a (4.2)	65.2 b (4.2)	31.1 a (7.5)	33.6 a (5.0)

* Mean values of three replicates (n=3); standard deviations are indicated in brackets. Mean values followed by different letters indicate significant differences ($p<0.05$) as a result of the icing condition.

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 3

Lipid oxidation development* in hake muscle stored under different icing conditions**

Chilling time (days)	**Peroxide value** (meq active oxygen kg^{-1} lipids)				**Thiobarbituric acid index** (mg malondialdehyde kg^{-1} muscle)				**Fluorescence ratio**			
	Control	AQ	ET	ET-AQ	Control	AQ	ET	ET-AQ	Control	AQ	ET	ET-AQ
0	1.11 (0.28)				0.25 (0.02)				0.32 (0.05)			
2	1.24 a (0.19)	1.55 a (0.07)	3.26 b (0.30)	3.57 b (0.16)	0.24 a (0.08)	0.36 a (0.08)	0.34 a (0.06)	0.45 a (0.11)	0.81 b (0.16)	0.72 b (0.11)	0.68 ab (0.26)	0.48 a (0.05)
6	0.89 a (0.47)	2.19 b (0.25)	3.61 c (0.60)	3.67 c (0.52)	0.37 a (0.10)	0.42 a (0.03)	0.48 a (0.06)	0.49 a (0.05)	0.81 b (0.14)	0.63 ab (0.10)	0.70 ab (0.15)	0.53 a (0.05)
9	3.00 a (0.39)	3.35 a (0.31)	6.41 b (1.10)	5.32 b (0.72)	0.63 b (0.08)	0.52 ab (0.11)	0.42 a (0.03)	0.55 ab (0.10)	0.67 b (0.10)	0.59 b (0.03)	0.66 b (0.04)	0.51 a (0.01)
13	1.37 b (0.20)	0.74 a (0.22)	2.95 c (0.46)	1.92 bc (0.51)	0.98 b (0.11)	1.02 b (0.35)	0.70 ab (0.11)	0.52 a (0.11)	0.76 a (0.27)	0.82 a (0.16)	0.68 a (0.15)	0.67 a (0.21)

* Mean values of three replicates (n=3); standard deviations are indicated in brackets. Mean values followed by different letters indicate significant differences ($p<0.05$) as a result of the icing condition.

** Abbreviations of icing conditions as expressed in Table 1.

Figure 1

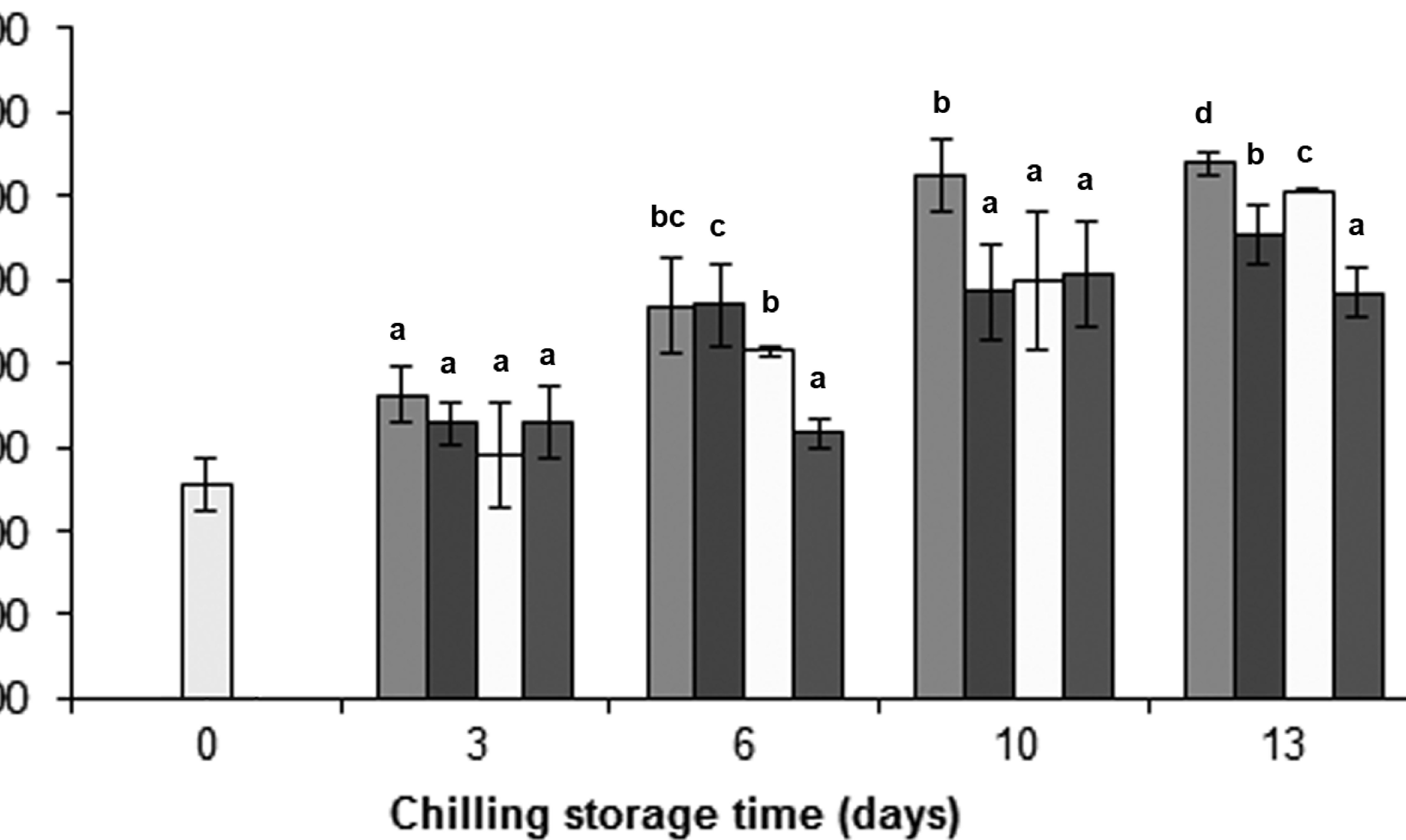

Figure 2