Effect of an icing medium containing the alga *Fucus spiralis* on the microbiological activity and lipid oxidation in chilled megrim (*Lepidorhombus whiffiagonis*)

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ABSTRACT

The present study provides a first approach on the employment of an icing medium including *Fucus spiralis*, an alga exhibiting antimicrobial and antioxidant activities, for the preservation of fish quality during chilled storage. For it, two different concentrations of a *F. spiralis* extract (0.67 and 2.50 g lyophilized alga L^{-1} aqueous solution; F-1 and F-2 batches, respectively) were tested as icing medium for the chilled storage of megrim (*Lepidorhombus whiffiagonis*) for 14 days. The effects of the alga were compared with a counterpart batch stored in traditional ice prepared only from water (F-0 batch). A significant ($p<0.05$) inhibition of microbial activity (aerobes, psychrotrophs, proteolytic bacteria, lipolytic bacteria; pH and trimethylamine formation) in F-1 batch and, especially in F-2 batch, was concluded. Concerning lipid oxidation development, a significantly ($p<0.05$) lower formation of interaction compounds (fluorescence assessment) in samples corresponding to the F-2 batch was also observed, proving the inhibitory effect of *F. spiralis* on the formation of tertiary lipid oxidation compounds in chilled megrim. The icing medium proposed in this study may open the way to the development of a natural biopreservation strategy for chilled seafood based on algae.

Keywords: *Lepidorhombus whiffiagonis*; *Fucus spiralis*; chilling; microbiological activity; lipid oxidation; quality.

Head Tittle: Biopreservation of chilled megrim with *Fucus spiralis*.

53 # 1. INTRODUCTION

54 With a view to slow down fish damage during chilled storage, thus extending its shelf
55 life, a wide number of preservation strategies have been combined with flake ice.
56 Among them, several chemical (phenolic compounds, bisulphites, polyphosphates, etc.)
57 and physical (namely, irradiation and high-pressure processing) treatments (Ashie,
58 Smith, & Simpson, 1996; Briones, Reyes, Tabilo-Munizaga, & Pérez-Won, 2010) and
59 the combination with packaging (Sivertsvik, Jeksrud, & Rosnes, 2002; González-
60 Fandos, Villarino-Rodríguez, García-Linares, García-Arias, & García-Fernández, 2005)
61 have been reported. Additionally, recent studies accounted for the incorporation of
62 preservative compounds such as ozone (Pastoriza, Bernárdez, Sampedro, Cabo, &
63 Herrera, 2008), vegetable extracts (namely, thyme, rosemary and oregano) (Oral,
64 Gülmez, Vatansever, & Güven, 2008; Quitral, Donoso, Ortiz, Herrera, Araya, &
65 Aubourg, 2009; Özyurt, Kuley, Balikçi, Kaçar, Gökdogan, Etyemez, & Özogul, 2012)
66 and natural low-molecular weight organic acids (Sallam, 2007; García-Soto, Fernández-
67 No, Barros-Velázquez, & Aubourg, 2014a) in the icing medium.

68 Marine algae are known to be part of the diet in different Asiatic countries
69 (namely, Japan, China and Korea) and constitute a source of beneficial nutrients, such
70 as vitamins, trace minerals, lipids, amino acids and dietary fibres (Díaz-Rubio, Pérez-
71 Jiménez, & Saura-Calixto, 2009; Paiva, Lima, Ferreira Patarra, Neto, & Baptista, 2014).
72 Their major use in Western countries has concentrated on the extraction of compounds
73 used in pharmaceutical, cosmetics and food industries (Smit, 2004; Gyawali & Ibrahim,
74 2014). Recently, red, green and brown macroalgae have offered the possibility of
75 exploring a wide variety of natural compounds with potential antioxidant
76 (Halldorsdóttir, Sveinsdóttir, Gudmundsdóttir, Thorkelsson, & Kristinsson, 2014) and
77 antimicrobial (Sandsdalen, Haug, Stensvag, & Styrvold, 2003) activities susceptible to

78 be applied to seafood. In this sense, a wide number of preservative metabolites such as
79 polyphenols, terpenes, phlorotannins, steroids, halogenated ketones and alkanes,
80 fucoxanthin, polyphloroglucinol or bromophenols have been isolated from macroalgae
81 (Fleurence, Morançais, Dumay, Decottignies, Turpin, Munier, García-Bueno, & Jaouen,
82 2012; Peinado, Girón, Koutsidis, & Ames, 2014). As a result, previous research
83 accounts for a preservative effect of alga extracts on seafood such as chilled cod muscle
84 (Wang, Jónsdóttir, Kristinsson, Thorkelsson, Jacobsen, Yuca Hamaguchi, & Olafsdóttir,
85 2010), canned Atlantic salmon (Ortiz, Vivanco, & Aubourg, 2014) and fish-oil enriched
86 food (Hermund, Iltas, Honold, Jónsdóttir, Kristinsson, & Jacobsen, 2015).

87 Flatfish (e.g., flounder, sole, turbot, plaice and halibut) species represent a very
88 important seafood group. Among them, megrim (*Lepidorhombus whiffiagonis*) is
89 abundant in the Northeast Atlantic waters. This species, which is considered one of the
90 most fished species in the Grand Sole North Atlantic Fishing bank, has been exploited
91 by a wide number of European countries, these including the United Kingdom, France,
92 Ireland and Spain (FAO, 2007). Previous studies on megrim have evaluated the effects
93 of different on-board storage conditions on chilled fish quality (Jehanno, Thuault,
94 Larnaud, Alaume, & Bourgeois, 1996; Aubourg, Losada, Gallardo, Miranda, & Barros-
95 Velázquez, 2006). Moreover, the evolution of different damage pathways in megrim has
96 been analysed, these including volatile amine formation during refrigeration (1-3ºC)
97 (Civera, Turi, Bisio, Parisi, & Fazio, 1995), microbial activity (Sanjuás-Rey, García-
98 Soto, Fuertes-Gamundi, Aubourg, & Barros-Velázquez, 2012) and lipid oxidation
99 (García-Soto, Sanjuás-Rey, Barros-Velázquez, Fuertes-Gamundi, & Aubourg, 2011)
100 during chilled storage. These reports underline the need of additional studies for the
101 development of natural refrigeration systems to meet the increasing consumer demand
102 for natural, high quality and safer seafood products.

Accordingly, this study evaluates the inclusion, to the best of our knowledge for the first time, of an alga extract in the icing medium employed for the chilled storage of a marine species. According to European Council Regulation (1997) algae are considered food or food ingredients, so their use in the icing medium should not constitute any hazard to health. *F. spiralis* was chosen for this study in agreement with its abundance in the Galician Atlantic coast (North-western Spain) and its promising biopreservation properties revealed recently by other authors (Andrade, Barbosa, Pedro Matos, Lopes, Vinholes, Mouga, & Valentão, 2013; Tierney, Smyth, Hayes, Soler-Vila, Croft, & Brunton, 2013a). For it, aqueous solutions including ethanolic extracts of lyophilised *F. spiralis* were employed at two different concentrations as icing media. The effects of the alga extracts on microbial activity inhibition and lipid oxidation stability were monitored in megrim muscle for up to 14 d of chilled storage by means of microbiological and biochemical analyses.

2. MATERIAL AND METHODS

2.1. Preparation of *Fucus spiralis* extracts and icing systems

The lyophilised alga *F. spiralis* was provided by Porto-Muiños (Cerceda, A Coruña, Spain). Fifteen g of lyophilised alga were mixed with absolute ethanol (2 x 120 mL), stirred for 30 s and centrifuged at 3500 rpm for 10 min at 4ºC. Then, the supernatant was recovered and diluted to 6 L with distilled water (2.50 g lyophilised alga L^{-1} aqueous solution). This solution was packaged in polyethylene bags, kept frozen at -18ºC and later used as icing medium (F-2 batch). In the same way, 4 g of lyophilised alga were extracted with ethanol as described above in order to provide a more diluted alga-icing medium (0.67 g lyophilised alga L^{-1} aqueous solution; F-1 batch). Finally, traditional ice was prepared from distilled water that was packaged and kept frozen in

128 the same way as the two other ices (F-0, control batch). Before addition to individual
129 fish specimens, the different icing systems were ground to obtain ice flakes.

130 Experimental conditions (content of lyophilised alga extract in the ice) employed
131 in the present study were based on several preliminary tests carried out at our laboratory
132 in the 0.2-10.0 g lyophilised alga L^{-1} aqueous solution range. Thus, an increasing
133 presence of alga in the icing medium provided better sensory acceptance. However a
134 concentration of 2.50 g L^{-1} resulted in the highest concentration without modifying the
135 sensory descriptors (external odour and colour, as well as flesh odour and flavour) of
136 the fish (data not shown). Consequently, this concentration was considered in the F-2
137 batch, together with a less concentrated concentration (0.67 g L^{-1}, F-1 batch).

138

139 **2.2. Fish material, processing and sampling**

140 Fresh megrim (117 specimens) were caught near the Galician Atlantic coast (North-
141 Western Spain) and transported to the laboratory. Throughout this process (10 h), the
142 fish were maintained in ice. The length and weight of the fish specimens ranged from
143 23 to 27 cm and from 110 to 137 g, respectively.

144 Upon arrival to the laboratory, nine individual fish specimens were separated
145 and analysed as initial fish (day 0). These fish specimens were divided into three
146 different groups (three individuals per group) that were analysed independently to
147 achieve the statistical analysis (n=3). The remaining fish specimens were divided into
148 three batches (36 individuals in each batch), that were placed in independent boxes and
149 directly surrounded by different kinds of ice (F-0, F-1 and F-2 batches, respectively),
150 prepared as previously described. Ice was added at a 1:1 fish:ice ratio, and all batches
151 were placed inside a refrigerated room (2±1ºC). Boxes that allowed draining of melted
152 ice were used for fish storage. The ice of all batches was renewed when required in

order to maintain the 1:1 fish:ice ratio. Fish samples from all of the batches were stored for a 14-day period, being sampled and analysed on days 4, 7, 11 and 14. At each sampling time, nine specimens were taken from each batch for analysis and divided into three groups (three individuals in each group) that were studied independently (n=3).

2.3. Microbiological analyses

Samples of 10 g of fish white muscle were taken aseptically from chilled fish specimens, mixed with 90 mL of 0.1% peptone water (Merck, Darmstadt, Germany) and homogenised in sterilised stomacher bags (AES, Combourg, France) as previously described (Ben-Gigirey, Vieites Baptista de Sousa, Villa, & Barros-Velázquez, 1998; Ben-Gigirey, Vieites Baptista de Sousa, Villa, & Barros-Velázquez, 1999). Serial dilutions from the microbial extracts were prepared in 0.1% peptone water.

Total aerobes were investigated by plating on plate count agar (PCA, Oxoid Ltd., London, UK) after incubation at 30ºC for 48 h. The anaerobe counts were also determined in PCA at 30±0.5ºC, except that an anaerobic atmosphere kit (Oxoid Ltd.) was placed inside the anaerobiosis jar. Psychrotrophs were also investigated in PCA, being the incubation carried out at 7-8ºC for 7 days. Enterobacteriaceae were investigated by pour plating using Violet Red Bile Agar (VRBA) (Merck, Darmstadt, Germany) after an incubation period of 24 h at 37±0.5ºC. Microorganisms exhibiting a proteolytic or lipolytic phenotype were determined on casein-agar medium or tributyrine-agar, respectively, after incubation at 30ºC for 48 h, as previously described (Ben-Gigirey, Vieites Baptista de Sousa, Villa, & Barros-Velázquez, 2000).

In all cases, microbial counts were transformed into log CFU g^{-1} muscle before undergoing statistical analysis. All of the analyses were conducted in triplicate.

177 **2.4. Chemical analyses**

178 Total polyphenols content of lyophilised *F. spiralis* was assessed by means of the Folin-
179 Ciocalteu colorimetric method (Cary 3E UV–Visible spectrophotometer, Varian;
180 Mulgrave, Victoria, Australia) as described previously (Rodríguez-Bernaldo de Quirós,
181 Frecha-Ferreiro, Vidal-Pérez, & López-Hernández, 2010). Measurements were made in
182 triplicate. Gallic acid (GA) was used as standard. Results were expressed as mg GA g^{-1}
183 lyophilised alga.

184 Chemical analyses related to fish quality were carried out on the white muscle of
185 megrim. All solvents and chemical reagents used were of reagent grade (Merck,
186 Darmstadt, Germany).

187 The evolution of pH values in megrim muscle along storage time was
188 determined using a 6-mm diameter insertion electrode (Crison, Barcelona, Spain).

189 Trimethylamine-nitrogen (TMA-N) values were determined using the picrate
190 colorimetric (Beckman Coulter, DU 640; London, UK) method, as previously described
191 by Tozawa, Erokibara, & Amano (1971). This method involves the preparation of a 5%
192 trichloroacetic acid extract of fish muscle (10 g/25 mL). The results are expressed as mg
193 TMA-N kg^{-1} muscle.

194 Lipids were extracted from the fish muscle by the Bligh & Dyer (1959) method,
195 which employs a single-phase solubilisation of the lipids using a chloroform-methanol
196 (1:1) mixture. The results were calculated as g lipid kg^{-1} muscle.

197 Free fatty acid (FFA) content was determined in the lipid extract of the fish
198 muscle by the Lowry & Tinsley (1976) method based on complex formation with cupric
199 acetate-pyridine followed by spectrophotometric (715 nm) assessment. Results were
200 expressed as g FFA kg^{-1} lipids and as mg FFA kg^{-1} muscle.

201 The peroxide value (PV) was determined spectrophotometrically (Beckman
202 Coulter, DU 640; London, UK) using the lipid extract via previous peroxide reduction
203 with ferric thiocyanate according to the Chapman & McKay (1949) method. The results
204 are expressed as meq active oxygen kg^{-1} lipids.
205 The thiobarbituric acid index (TBA-i) was determined according to Vyncke
206 (1970). This method is based on the reaction between a trichloracetic acid extract of the
207 fish muscle and thiobarbituric acid. Content of thiobarbituric acid reactive substances
208 (TBARS) was spectrophotometrically measured at 532 nm and calculated from a
209 standard curve using 1,1,3,3-tetraethoxy-propane (TEP); results were expressed as mg
210 malondialdehyde kg^{-1} muscle.
211 Tertiary lipid oxidation compounds resulting from the interaction between
212 oxidised lipids and nucleophilic compounds (namely, protein-like molecules) were
213 measured by fluorescence spectroscopy. Such measurement has shown to be a valuable
214 tool for assessing the fish quality changes during processing (Losada, Piñeiro, Barros-
215 Velázquez, & Aubourg, 2004). Thus, the formation of fluorescent compounds
216 (Fluorimeter LS 45; Perkin Elmer España; Tres Cantos, Madrid, Spain) was determined
217 by measurements at 393/463 nm and 327/415 nm. The relative fluorescence (RF) was
218 calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each
219 excitation/emission maximum and F_{st} is the fluorescence intensity of a quinine sulphate
220 solution (1 µg mL^{-1} in 0.05 M H_2SO_4) at the corresponding wavelength. The
221 fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$. The FR value was determined using the aqueous phase that
223 resulted from the lipid extraction of the fish muscle (Bligh & Dyer, 1959).
224

225 **2.5. Statistical analysis**

226 Data obtained from the different microbiological and chemical analyses were subjected
227 to the ANOVA method to explore differences resulting from the effects of both the
228 icing condition and the chilling time; the comparison of means was performed using the
229 least-squares difference (LSD) method. In all cases, analyses were carried out using the
230 PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences
231 among batches and among refrigeration times were considered significant for a
232 confidence interval at the 95% level ($p<0.05$) in all cases.

233 Correlation analysis among parameters (chilling storage time, microbiological
234 values and chemical indices) was also carried out. In it, linear fittings are mentioned;
235 otherwise, the kind of fitting is expressed.

236

237 **3. RESULTS AND DISCUSSION**

238 **3.1. Analysis of the microbial development in megrim**

239 Preliminary plate bioassays carried out at our laboratory showed that ethanolic
240 extracts of *F. spiralis* exhibited antimicrobial activity against *Bacillus cereus, Bacillus*
241 *subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas fluorescens,*
242 *Escherichia coli, Aeromonas hydrophila, Vibrio alginolyticus* and *Vibrio*
243 *parahaemolyticus* (data not shown). Additionally, a high polyphenol content (53.3±5.0
244 GA g^{-1} lyophilised alga) was determined in ethanolic extracts of *F. spiralis*.

245 The results of the investigation of aerobic mesophiles in all three batches are
246 compiled in Table 1. A progressive increase was observed for all kinds of samples
247 throughout the chilling storage time (r^2 = 0.93-0.95, quadratic fitting). The inclusion of
248 *F. spiralis* extract in the icing medium provided a better control of aerobes as compared
249 with the control batch (F-0), the microbial inhibition reaching its maximum (1.25 log

250 units) at advanced storage periods. This was especially remarkable in the case of the F-2
251 batch, which included a higher concentration of *F. spiralis* extract. Thus, statistically
252 significant ($p<0.05$) differences were observed between the control batch (F-0) and F-2
253 batch after 11 and 14 d of storage. In contrast, F-1 batch provided an intermediate
254 protection against aerobes growth as compared with F-2 batch, and no significant
255 ($p>0.05$) differences were observed between F-1 and F-0 batches (Table 1).

256 The development of psychrotrophs in megrim batches is also presented in Table
257 1. Psychrotrophs normally include bacteria belonging to genera *Pseudomonas,*
258 *Moraxella, Acinetobacter, Shewanella and Flavobacterium*, some of them being of
259 concern for the preservation of microbial fish quality. The evolution of psychrotrophs
260 followed a similar behaviour as that observed for aerobes. Thus, a progressive increase
261 was observed for all kinds of samples throughout the chilling storage time (r^2 = 0.93-
262 0.95). The batches including *F. spiralis* extracts showed slight decreases of
263 psychrotrophs counts as compared with the control batch, these reductions being more
264 remarkable for F-2 batch at advanced storage times. Thus, significantly ($p<0.05$) lower
265 psychrotrophs counts were determined in the F-2 batch on day 11, such differences
266 reaching 0.89 log units at that storage time. This result, as in the case of aerobes,
267 indicates a positive effect derived from the inclusion of *F. spiralis* extract in the icing
268 medium in terms of microbial inhibition in megrim muscle.

269 The investigation of Enterobacteriaceae (Table 1) provided similar results in all
270 three batches. Remarkably, the numbers of this microbial group were very low in all
271 batches at all storage times, a result that confirms the good initial microbial quality of
272 megrim specimens. Thus, numbers below 1 log CFU g^{-1} were determined in all batches
273 even after 11 d of storage. As a consequence of this, no significant ($p>0.05$) differences
274 among batches could be concluded.

The evolution of proteolytic microorganisms in all three batches is presented in Figure 1. The results obtained for this microbial group were in agreement with those observed for aerobes and psychrothrophs. Thus, a progressive increase ($p<0.05$) was observed for all kinds of samples throughout the chilling storage time (r^2 = 0.92-0.94). The presence of *F. spiralis* extracts in the icing medium, especially in the case of the F-2 batch, provided a significant ($p<0.05$) protection against this microbial group at advanced storage times. The microbial inhibition reached its maximum (1.01 log units) on day 11 (Figure 1). The crucial role of proteolytic microorganisms in the degradation of fish muscle has been previously reported (Rodríguez, Barros-Velázquez, Ojea, Piñeiro, & Aubourg, 2003). Accordingly, the partial inhibition of the action of this microbial group due to the presence of *F. spiralis* extracts in the icing medium tested in this study is another remarkable result in terms of fish quality.

Figure 2 compiles the results of the investigation of lipolytic bacteria in all three batches. A progressive increase ($p<0.05$) was observed for all kinds of samples throughout the chilling storage time (r^2 = 0.89-0.93). Significant ($p<0.05$) differences among F-2 and F-0 batches were observed after 4 d of storage. Remarkably, such differences reached maximum values of 1.54 log CFU g^{-1} after 11 d of storage, these being the most marked differences among batches determined for any microbial group.

The antimicrobial effect observed in this study on megrim for aerobes, psychrothrophs, proteolytic and lipolytic bacteria, especially at advanced storage times, is a consequence of the incorporation of *F. spiralis* ethanolic extracts in the icing medium. Chemicals responsible for preservative activities are widespread in macroalgae. The antimicrobial effect of *Fucus* spp. and other algae has been linked to the presence of terpenes and polyphenols (Sandsdalen et al., 2003) and oligomeric phlorotannins (Wang et al., 2010), although other compounds might also be involved

300 (Fleurence et al., 2012; Peinado et al., 2014). In a closely related alga species (*Fucus*
301 *vesiculosus*), Sandsdalen et al. (2003) identified by electrospray ionization mass
302 spectrometry a polyhydroxylated fucophlorethol as being responsible for antimicrobial
303 activity against both the Gram-positive and Gram-negative bacteria tested. Interesting
304 antimicrobial substances in particular are the halogenated compounds such as
305 haloforms, halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones and
306 ketones (Smit, 2004). Some of them have been applied for their effectiveness as active
307 ingredient in bacterial antifouling (Kjelleberg & Steinberg, 2001).

308 Other authors have also reported that the inclusion of natural compounds in the
309 icing system can reduce the microbial activity. Among them, the following two should
310 be remarked: (i) the inclusion of a rosemary extract in the icing medium and its
311 application to the chilled storage of sardines (*Sardinella aurita*) (Özyurt et al., 2012),
312 and (ii) the incorporation of wild-thyme hydrosol extract to the chilling medium of
313 Transcaucasian barb (*Capoeta capoeta capoeta*) (Oral et al., 2008). However, the
314 present study represents, to our knowledge, the first report of *F. spiralis* extract in the
315 icing medium for the preservation of fish.

316 This work, carried out on megrim, also complements previous reports focused
317 on the preservation of this fish species under other advanced refrigeration systems, such
318 as slurry ice (Aubourg et al., 2006) or addition of ozone to the icing medium (Pastoriza
319 et al., 2008). Slurry ice slowed down microbial growth in fish muscle due to the
320 washing effect of the salt solution on the fish surface during ice melting (Losada et al.,
321 2004; Campos, Rodríguez, Losada, Aubourg, & Barros-Velázquez, 2005). In the
322 present work, the melting of the flake ice crystals containing *F. spiralis* extract might
323 exert a similar washing effect that may reduce the surface microbial load, thus limiting
324 its diffusion towards megrim muscle.

325 Natural organic acids, such as citric acid and lactic acid, have previously been
326 used for the preparation of flake ice used for megrim storage with good results (García-
327 Soto, Böhme, Barros-Velázquez, & Aubourg, 2014b). Likewise, the inclusion of
328 ascorbic acid, combined with citric and lactic acids, has also been reported to provide
329 control of aerobic mesophiles in megrim as well as in hake (*Merluccius merluccius*) and
330 angler (*Lophius piscatorius*) (Sanjuás-Rey et al., 2012).
331
332 **3.2. Analysis of the chemical changes**
333 Two chemical quality parameters (namely, pH and TMA-N values) closely related to
334 the microbial activity development were studied.
335 Concerning the pH value, a significant ($p<0.05$) and progressive increase was
336 observed for all kinds of samples along chilling storage time (r^2 = 0.90-0.95, quadratic
337 fitting) (Figure 3). An inhibitory effect on pH increase was observed as a result of
338 including the lyophilised alga in the icing medium. At all storage times, analysis of the
339 mean values of the different fish-sample groups led to the following pH increasing
340 sequence: F-2 < F-1 < F-0, so that a 7.18 score was obtained for control fish at the end
341 of the study. Differences between control and alga-treated fish were found significant
342 ($p<0.05$) at days 7 and 14. Comparison among both alga concentrations showed lower
343 mean values in the F-2 batch, being the difference significant ($p<0.05$) at day 4.
344 Increases in the pH value of fish muscle indicate the accumulation of alkaline
345 compounds, such as ammonia, TMA and other nitrogen-including compounds, which
346 are mainly derived from microbial activity. Thus, pH values showed good correlation
347 values with microbiological parameters (r^2 = 0.76-0.95), the best scores being observed
348 for aerobe counts (r^2 = 0.89-0.95). In agreement with these results, previous studies have
349 reported an inhibition of pH increase as a result of using other natural preservative

350 compounds in ice during the chilled storage of other marine species. These studies
351 included the use of oregano and rosemary extracts during the chilled storage of Chilean
352 jack mackerel (*Trachurus murphyi*) (Quitral et al., 2009), a rosemary extract applied to
353 sardine (*Sardinella aurita*) (Özyurt et al., 2012) and a wild-thyme hydrosol extract
354 employed for the chilled storage of Transcaucasian barb (Oral et al., 2008).

355 A progressive formation of TMA was observed as storage time progressed (r^2 =
356 0.88-0.94, quadratic fitting) in all batches (Table 2). As a result of this, notably high
357 TMA contents were determined at the end of the experiment, this being in agreement
358 with previous studies on megrim chilled storage (Civera et al., 1995; Aubourg et al.,
359 2006; Sanjuás-Rey et al., 2012). Comparison among batches allowed to conclude a
360 significant ($p<0.05$) inhibitory effect at days 7 and 11 in the F-2 batch.

361 Volatile amine compounds are produced partially by means of endogenous
362 enzyme activity but mostly as a result of microbial development (Ashie et al., 1996;
363 Campos, Gliemmo, Aubourg, & Barros-Velázquez, 2012). Among them, TMA is one of
364 the most commonly employed quality methods to assess microbial activity in marine
365 species kept under refrigerated conditions. In agreement with this close relationship
366 with microbial activity, TMA values showed good correlation values with aerobe,
367 psychrotroph and proteolytic counts (r^2 = 0.77-0.91), showing the best scores when
368 compared with the aerobe counts (r^2 = 0.88-0.91). Additionally, a good correlation was
369 obtained with the pH value (r^2 = 0.84-0.90).

370 A previous report (Sanjuás-Rey et al., 2012) showed that the employment of a
371 commercial formula including natural organic acids (lactic, citric and ascorbic) in the
372 icing system did not produce any significant improvement in terms of TMA formation.
373 However, if citric and lactic acids were directly included in the icing medium, a small
374 inhibitory effect on TMA formation was observed (García-Soto et al., 2014a; García-

375 Soto et al., 2014b). Additionally, the application of other preservative strategies such as
376 slurry ice (Aubourg et al., 2006) and ozonised ice (Pastoriza et al., 2008) has shown to
377 inhibit TMA formation in chilled fish.

378 Lipid hydrolysis was measured by means of the FFA content (Table 2). This
379 parameter revealed a progressive formation in all cases throughout storage time (r^2 =
380 0.89-0.93, quadratic fitting). Comparison among the different samples under study did
381 not provide a definite trend concerning the effect of *F. spiralis* on lipid hydrolysis.
382 Thus, a higher FFA formation ($p<0.05$) was observed at advanced storage (11-14 days)
383 in F-1 batch as compared to F-0 batch. On the other hand, higher mean values were
384 determined in the control batch in the 7-14-day period as compared with the alga-
385 concentrated fish group (F-2 batch). Similar conclusions were obtained when FFA
386 content is considered on muscle basis (mg kg^{-1} muscle). In this case, FFA formation
387 also showed a good correlation value with the storage time in all cases (r^2 = 0.88-0.92,
388 quadratic fitting).

389 Although the formation of FFA itself does not lead to nutritional losses,
390 accumulation of FFA has been related to some extent to lack of acceptability, since FFA
391 are known to have detrimental effects on protein properties (Sikorski & Kolakowska,
392 1994) and oxidise faster than higher-molecular weight lipid classes (namely,
393 triglycerides and phospholipids) by providing a greater accessibility (lower steric
394 hindrance) to oxygen and other pro-oxidant molecules. FFA formation during chilling
395 storage has been reported to be produced as a result of both endogenous enzyme activity
396 and microbial activity (Ashie et al., 1996; Campos et al., 2012). Before the end of the
397 microbial lag phase (up to 6-9 days, depending on several factors), FFA formation
398 should be produced mostly as a result of endogenous enzyme (namely, lipases and
399 phospholipases) activity. Later on, microbial activity should gain importance, so that

400 FFA formation should be produced especially as a result of bacterial catabolic
401 processes. The fact that a definite trend of the effect of *F. spiralis* on FFA content was
402 not found in the present work could be explained by the fact that different FFA
403 formation mechanisms are involved. FFA values determined in the present study
404 showed good correlation values with some indices related to microbial activity, such as
405 aerobe counts (r^2= 0.86-0.93), pH value (r^2= 0.86-0.92) and TMA-N content (r^2= 0.92-
406 0.94).

407 In agreement with the present results, an inhibitory effect of a rosemary extract
408 on lipid hydrolysis in chilled sardine was not observed by Özyurt et al. (2012). In
409 contrast, an inhibitory effect on fish FFA formation could be observed by the
410 application of a commercial formula including organic acids (citric, lactic and ascorbic)
411 in the icing medium during megrim storage (García-Soto et al., 2011) as well as by
412 including a rosemary and oregano extract to the preservation of chilled Chilean jack
413 mackerel (Quitral et al., 2009). In addition, a lower FFA formation was observed in blue
414 whiting (*Micromesistius poutassou*) muscle after a two-step process that consisted of
415 dipping and icing fish with the above-mentioned formula (Sanjuás-Rey, García-Soto,
416 Barros-Velázquez, Fuertes-Gamundi, & Aubourg, 2011).

417 Primary and secondary lipid oxidation development in megrim muscle (3.9-5.1 g
418 lipids kg^{-1} muscle) was measured by assessment of the peroxide and TBARS content,
419 respectively (Table 3). Formation of peroxides throughout storage time provided, with
420 some exceptions, an increase with storage time, although correlation values were not
421 satisfactory. This increase was especially marked in all cases at the end of the storage
422 time. Comparison among batches showed higher values in fish specimens
423 corresponding to the F-2 batch throughout the 4-14-day period. In contrast, the less-
424 concentrated alga batch (F-1) showed higher mean values in the 11-14-day period when

compared with control fish. Consequently, an enhancement effect on peroxide content can be concluded by the presence of alga extract in the icing medium.

Concerning secondary lipid oxidation, TBARS formation can be considered as very low, being included all scores in the 0.22-0.44 range. TBARS content increased with storage time although correlation was poor. Comparison among batches provided only slight differences, so that a general trend of the effect of *F. spiralis* on TBARS formation in megrim muscle could not be concluded.

Tertiary lipid oxidation was assessed by means of fluorescent compounds formation (Table 3). With some exceptions, a general increase was observed as storage time progressed (r^2= 0.80-0.90, quadratic fitting). Such increase was especially relevant in all batches at day 11. Comparison among the different samples showed lower mean FR values in samples corresponding to the F-2 batch in the 11-14-day period, being differences significant ($p<0.05$) at the end of the storage time. Consequently, an inhibitory effect of *F. spiralis* on tertiary lipid oxidation compounds formation could be concluded. This inhibitory effect is in agreement with the high level of polyphenol compounds (53.3±5.0 GA g^{-1} lyophilised alga) determined in the ethanolic extracts of *F. spiralis*.

Lipid oxidation has been recognised as a complex process where different kinds of molecules are produced, most of them unstable, susceptible to breakdown and to originate lower-weight compounds, or to react with other molecules (nucleophilic-type, mostly) present in the fish muscle. As a result of this, determination of each kind of compound cannot always provide an accurate method for the quality loss assessment in fish. In the present study, primary and secondary lipid oxidation compounds did not provide a satisfactory correlation with storage time. The electrophilic nature of such compounds led them to interact with food constituents possessing nucleophilic

functions (Losada et al., 2004; Campos et al., 2012), so that FR assessment provided the best correlation value with storage time, this being in agreement with previous studies on chilled megrim (Aubourg et al., 2006; García-Soto et al., 2011). Additionally, FR values showed good correlation scores with aerobe counts (r^2= 0.81-0.90), and fair with other chemical parameters such as pH value (r^2= 0.76-0.85), TMA-N value (r^2= 0.61-0.92) and FFA content (r^2= 0.63-0.93).

The antioxidant capacity and reducing ability of ethanolic extracts of alga *F. spiralis* were previously reported by means of in-vitro DPPH and FRAP tests (Andrade et al., 2013; Peinado et al., 2014). This alga showed a marked content on polyphenols (90-205 μg phloroglucinol equivalents mg^{-1}) (Tierney et al., 2013a) and α-tocopherol (511.4 mg kg^{-1}) (Paiva et al., 2014). Additionally, a preliminary identification of active compounds was carried out by quadrupole time-of-flight mass spectrometry (Q-TOF-MS) (Tierney, Smyth, Rai, Soler-Vila, Croft, & Brunton, 2013b); as a consequence, the results supported the assumption that pholorotannins were present and were probably involved in the antioxidant activity.

Previous studies have shown that including natural preservative compounds in the icing system provides an inhibitory effect on fish lipid oxidation. Directly related to the present work, the employment of a commercial formula including citric, lactic and ascorbic acids led to a lower FR score during the chilled storage of megrim and hake (García-Soto et al., 2011) and blue whiting (Sanjuás-Rey et al., 2011). The same result was obtained when a laboratory-scale solution of citric and lactic acids was employed as icing medium during megrim storage (García-Soto et al., 2014b). Additionally, primary and secondary lipid oxidation compounds formation was inhibited in Chilean jack mackerel as a result of including oregano and rosemary extracts in the icing system (Quitral et al., 2009) and rosemary extract in sardine icing system (Özyurt et al., 2012).

4. CONCLUSIONS

The development and application of an icing system including an ethanolic extract of the alga *F. spiralis* has shown a protective effect on refrigerated megrim quality, according to the assessment of microbial activity and lipid oxidation development in the fish muscle. The present study provides a first approach focused on the employment of this abundant alga species, whose preservative properties and components have already been described in *in vitro* experiments. The results presented in this work constitute a promising basis in order to apply algae extracts in the icing system to enhance fish quality retention during storage in the different steps (on-board, transport, retail market, etc.) included in the commercialisation of refrigerated marine species.

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658
659

660 **FIGURE LEGENDS**

661

662 **Figure 1**: Proteolytic bacteria (log CFU g^{-1} muscle) assessment* in chilled megrim
663 muscle stored under different icing conditions.
664 * Initial value: 3.39±0.18. Mean values of three replicates (n = 3); standard deviations
665 are indicated in brackets. For each chilling time, mean values followed by
666 different capital letters (A-B) indicate significant ($p<0.05$) differences as a result
667 of the icing condition. No letters are indicated when significant differences are
668 not found ($p>0.05$).

669

670 **Figure 2**: Lipolytic bacteria (log CFU g^{-1} muscle) assessment* in chilled megrim
671 muscle stored under different icing conditions.
672 * Initial value: 2.16±0.28. Mean values of three replicates (n = 3); standard deviations
673 are indicated in brackets. For each chilling time, mean values followed by
674 different capital letters (A-B) indicate significant ($p<0.05$) differences as a result
675 of the icing condition. No letters are indicated when significant differences are
676 not found ($p>0.05$).

677

678 **Figure 3**: Assessment* of the pH value in chilled megrim muscle stored under different
679 icing conditions.
680 * Initial value: 6.42±0.09. Mean values of three replicates (n = 3); standard deviations
681 are indicated in brackets. For each chilling time, mean values followed by
682 different capital letters (A-B) indicate significant ($p<0.05$) differences as a result
683 of the icing condition. No letters are indicated when significant differences are
684 not found ($p>0.05$).

TABLE 1

Microbiological count assessment (log CFU g^{-1})* in chilled megrim muscle stored under different icing conditions**

Chilling time (days)	**Aerobes**			**Psychrotrophs**			**Enterobacteriae**		
	F-0	F-1	F-2	F-0	F-1	F-2	F-0	F-1	F-2
0	3.25 a (0.24)	3.25 a (0.24)	3.25 a (0.24)	3.76 a (0.31)	3.76 a (0.31)	3.76 a (0.31)	< 1 a	< 1 a	< 1 a
4	3.80 ab (0.56)	3.90 ab (0.61)	3.23 a (0.58)	4.71 a (0.70)	5.00 b (0.06)	4.93 b (0.22)	< 1 a	1.10 a (0.70)	< 1 a
7	5.11 bc (0.72)	4.22 b (0.28)	4.19 b (0.24)	6.15 b (0.32)	5.77 bc (0.81)	5.67 c (0.34)	< 1 a	1.11 a (0.17)	< 1 a
11	6.35 cB (0.65)	5.96 cB (0.21)	5.25 cA (0.21)	7.28 cB (0.62)	6.99 cdB (0.37)	6.39 dA (0.04)	< 1 a	< 1 a	< 1 a
14	7.39 dB (0.11)	7.26 dAB (0.26)	6.72 dA (0.36)	7.42 c (0.47)	7.30 d (0.36)	7.25 e (0.21)	2.37 b (0.13)	2.73 b (0.38)	2.73 b (0.38)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different capital letters (A-B) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different low-case letters (a-e) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

** Abbreviations of icing conditions: F-0 (Control; no seaweed extract presence in ice), F-1 (ice prepared from a 0.67 g L^{-1} aqueous solution of *F. spiralis* extract) and F-2 (ice prepared from a 2.50 g L^{-1} aqueous solution of *F. spiralis* extract).

TABLE 2

Assessment* of trimethylamine-nitrogen (TMA-N) and free fatty acids contents in chilled megrim muscle stored under different icing conditions**

Chilling time (days)	TMA-N (mg TMA-N kg^{-1} muscle)			Free fatty acids content ($g\ kg^{-1}$ lipids)		
	F-0	F-1	F-2	F-0	F-1	F-2
0	1.5 a (0.7)	1.5 a (0.7)	1.5 a (0.7)	10.4 a (1.6)	10.4 a (1.6)	10.4 a (1.6)
4	5.4 b (2.0)	4.1 ab (2.3)	7.2 b (2.9)	10.4 a (0.1)	14.3 a (5.7)	12.0 ab (1.7)
7	45.2 cB (14.4)	7.2 bA (8.9)	18.1 bA (7.9)	26.3 b (8.4)	14.1 a (3.9)	17.1 bc (3.5)
11	100.8 dB (29.3)	180.4 cC (18.1)	58.3 cA (11.5)	27.5 bA (7.7)	63.7 bB (8.4)	21.1 cA (3.7)
14	198.3 e (17.5)	193.4 c (48.2)	240.7 d (50.6)	62.8 cA (12.4)	95.3 cB (11.9)	48.1 dA (13.7)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different capital letters (A-C) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different low-case letters (a-e) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

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

TABLE 3

Lipid oxidation assessment* in chilled megrim 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**		
	F-0	F-1	F-2	F-0	F-1	F-2	F-0	F-1	F-2
0	0.45 a (0.27)	0.45 a (0.27)	0.45 a (0.27)	0.22 a (0.03)	0.22 a (0.03)	0.22 a (0.03)	2.46 a (0.64)	2.46 a (0.64)	2.46 ab (0.64)
4	1.55 bA (0.62)	2.69 bcA (0.55)	5.33 cB (1.26)	0.24 ab (0.04)	0.35 ab (0.11)	0.24 a (0.05)	2.18 a (0.42)	1.91 a (0.35)	1.80 a (0.30)
7	1.38 bA (0.17)	0.92 aA (0.41)	2.67 bB (1.11)	0.34 abAB (0.15)	0.22 aA (0.02)	0.33 bB (0.01)	2.18 a (0.19)	2.25 a (0.50)	2.66 bc (0.27)
11	0.87 abA (0.72)	1.99 bB (0.27)	5.08 cC (1.05)	0.36 b (0.07)	0.34 b (0.05)	0.38 b (0.07)	4.06 b (0.41)	4.15 b (0.43)	3.66 d (0.09)
14	2.53 bA (1.54)	5.02 cA (2.00)	8.96 dB (0.84)	0.31 b (0.03)	0.44 b (0.10)	0.42 b (0.06)	4.77 bB (0.44)	5.03 bB (0.57)	3.45 cdA (0.49)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different capital letters (A-C) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different low-case letters (a-d) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

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

Figures 1-3

Chilling time (days)	Proteolitics (Fig 1)			Lipolitics (Fig 2)			pH (Fig 3)		
	F-0	F-1	F-2	F-0	F-1	F-2	F-0	F-1	F-2
4	3.98 a (0.26)	4.38 a (0.20)	4.10 a (0.23)	3.43 a (0.90)	3.84 a (0.11)	3.54 ab (0.52)	6.61 aAB (0.14)	6.58 aB (0.03)	6.44 aA (0.06)
7	5.13 bAB (0.60)	5.05 bB (0.25)	4.36 aA (0.21)	4.36 aB (0.47)	4.20 abB (0.42)	3.49 aA (0.25)	6.75 aB (0.02)	6.54 aA (0.05)	6.51 abA (0.07)
11	6.19 cB (0.13)	6.79 cB (0.49)	5.18 bA (0.22)	5.82 bB (0.12)	4.60 bA (0.24)	4.28 abA (0.48)	6.78 a (0.10)	6.71 b (0.03)	6.65 b (0.05)
14	6.61 dB (0.21)	6.53 cAB (0.58)	5.99 cA (0.24)	5.90 bB (0.10)	5.50 cB (0.54)	4.36 bA (0.42)	7.18 bB (0.06)	6.96 cA (0.08)	6.83 cA (0.07)