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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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14 Jorge Barros-Velázquez¹, José M. Miranda¹, J. Marina Ezquerro-Brauer²,
15 Santiago P. Aubourg^{3,*}
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23 ¹ Department of Analytical Chemistry, Nutrition and Food Science, School of
24 Veterinary Sciences, University of Santiago de Compostela, Lugo (Spain)

25 ² Departamento de Investigación y Posgrado en Alimentos, University of Sonora,
26 Hermosillo (Sonora, Mexico)

27 ³ Department of Food Science and Technology, Marine Research Institute (CSIC), Vigo
28 (Spain)

29 * Corresponding author. Phone: +34986231930; Fax: +34986292762; e-mail:
30 saubourg@iim.csic.es
31
32
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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.

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Preparation of *F. spiralis* extracts and icing systems

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

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

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

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

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

Fish material, processing and sampling

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

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

Microbiological analyses related to quality loss

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

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

For all kinds of analyses, microbial counts obtained were transformed into log CFU g⁻¹ muscle values before undergoing the statistical analysis. All analyses were carried out in triplicate.

Chemical analyses related to quality loss

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

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

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

Formation of tertiary lipid oxidation compounds was measured by fluorescence spectroscopy (Fluorimeter LS 45, Perkin Elmer España; Tres Cantos, Madrid, Spain) at 393/463 nm and 327/415 nm in the aqueous phase resulting from the lipid extraction of hake muscle (Aubourg *et al.*, 1995). The relative fluorescence (RF) was calculated according to: $RF = F/F_{st}$, where F is the fluorescence determined at each excitation/emission wavelength pair and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 µg mL⁻¹ in 0.05 M H₂SO₄) measured at the corresponding wavelength pair. Results are expressed as the fluorescence ratio (FR), which was determined as the ratio between the two RF values: $FR = RF_{393/463\text{ nm}}/RF_{327/415\text{ nm}}$.

Statistical analysis

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

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

RESULTS AND DISCUSSION

Quality evaluation by assessment of microbiological analyses

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

Remarkably, aerobe concentrations were above 6 log units in both Control and AQ batches after 12 d of storage, while ET and ET-AQ levels were below such level, usually considered as a limit of microbial acceptability of fish products.

The comparative evolution of psychrotrophs in hake batches is also shown in Table 1. Psychrotrophic bacteria include members of the genera *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella* or *Flavobacterium*, some of them being of concern for their role in fish spoilage. The evolution of psychrotrophs followed a progressive increase in all four batches as storage time progressed ($r^2 = 0.90-0.94$). As in the case of aerobes, the batches including the ethanolic extract showed lower mean values than their corresponding Control and AQ batches in advanced storage times (9-13-day period). Additionally, the ET-AQ extract exhibited a better control of psychrotrophs growth as compared with the AQ and Control batches, these reductions being significant ($p < 0.05$) on day 9 and reaching a maximum of 0.91 log units. These results, as those observed for the aerobes, indicate a benefit derived from the inclusion of ethanolic (ET batch) or ethanolic/aqueous (ET-AQ) *F. spiralis* extracts in the icing medium in terms of microbial inhibition in hake muscle. However, no differences ($p > 0.05$) could be concluded between ET-AQ and ET batches.

The evolution of Enterobacteriaceae counts (Table 1) did not reveal significant ($p > 0.05$) differences among all four batches. Thus, the counts of this microbial group were remarkably low in all batches at all sampling times, reaching levels in the range of 1.00-1.43 log units. These results confirm the very good initial microbial quality of the hake specimens considered in this study.

The investigation of microorganisms exhibiting a proteolytic phenotype is presented in Figure 1. The negative effect of proteolytic bacteria in the degradation and spoilage of fish muscle has been previously described (Rodríguez *et al.*, 2003). The

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

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

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

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

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

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

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

Quality evolution by assessment of chemical analyses

Concerning the pH value in hake muscle, a progressive and significant ($p < 0.05$) increase was obtained in all hake batches as storage time progressed ($r^2 = 0.82-0.93$, quadrating fitting) (Table 2). Comparison of samples corresponding to the different icing conditions did not lead to significant differences ($p > 0.05$). However, lower mean values were observed throughout the 6-13-day period in fish stored under ET-AQ condition when compared with their counterpart Control.

Increases in the pH value of fish muscle corresponds to the accumulation of alkaline compounds, such as trimethylamine, ammonia and other nitrogen-including compounds, which are mostly derived from microbial activity (Sandsdalen *et al.*, 2003). Thus, accurate correlation values were obtained in the present study between the pH value and the different microbial parameters, especially with the lipolytic counts ($r^2 =$

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

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

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

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

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

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

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

In agreement with their photosynthetic role, algae are known to be exposed to a strong combination of light and oxygen. Thus, their natural content on antioxidant substances has been reported to be responsible for the lack of structural damage in their organs (Smit, 2004). The antioxidant behaviour and reducing ability of ethanolic extracts of *F. spiralis* were previously proved by means of in-vitro DPPH and FRAP tests (Cérantola *et al.*, 2006; Andrade *et al.*, 2013; Peinado *et al.*, 2014). This alga showed a profitable 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). In a structural study (Cérantola *et al.*, 2006), *F. spiralis* showed the simultaneous production of two types of polymeric phlorotannins of the fucol and fucophloretol classes, respectively. Additionally, Tierney *et al.* (2013b) provided the identification of different active compounds by quadrupole time-of-flight mass spectrometry (Q-TOF-MS), this study supporting the assumption that phlorotannins present would be involved in the antioxidant behaviour. Further, an inhibitory effect on lipid oxidation development was demonstrated by its inclusion in a biodegradable packaging film (García-Soto *et al.*, 2015) and in the icing medium (Miranda *et al.*, 2016) employed during megrim (*L. whiffiagonis*) refrigeration.

In contrast with the results of the microbiological analyses, some differences concerning chemical quality between ET and ET-AQ batches could be observed in the 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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FIGURE LEGENDS

Figure 1: Proteolytic bacteria assessment* in chilled hake muscle stored under different icing conditions**.

* Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. For each chilling time, mean values accompanied by different letters (a, b) indicate significant ($p < 0.05$) differences as a result of the icing condition.

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

Figure 2: Lipolytic bacteria assessment* in chilled hake muscle stored under different icing conditions**.

* Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. For each chilling time, mean values accompanied by different letters (a, b) indicate significant ($p < 0.05$) differences as a result of the icing condition.

** 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