Effectiveness of a combined ethanol-aqueous extract of alga *Cystoseira compressa* for
the quality enhancement of a chilled fatty fish species

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ABSTRACT

Seaweeds have attracted an increasing attention as a new source for bioactive compounds, these including preservative compounds. This study is a first attempt to employ alga *Cystoseira compressa* for the preservation of chilled fish. For it, a combined ethanol-aqueous extract of this alga was included in the icing system and employed as chilling medium for the storage of horse mackerel (*Trachurus trachurus*) during 11 days. On the basis of the microbial groups (aerobes, psychrotrophs, proteolytic, lipolytic and Enterobacteriaceae bacteria) assessed, an inhibitory effect ($p<0.05$) on microbial activity in horse mackerel muscle was observed as a result of including the alga extract in the icing medium; such preservative effect was also proved by chemical determinations related to microbial activity (pH and trimethylamine values). Additionally, a significant decrease ($p<0.05$) of lipid hydrolysis (free fatty acids formation) and oxidation (fluorescent compounds formation) in fish was also observed as a result of the presence of *C. compressa* extracts in the icing medium. The icing medium proposed in this work (i.e., combination of ethanolic and aqueous extracts of *C. compressa*) may constitute a promising strategy to the application of natural algae extracts for fatty fish storage and enhance the quality retention during commercialisation.

Keywords: *Cystoseira compressa*; ethanolic and aqueous extracts; *Trachurus trachurus*; chilling; antimicrobial activity; lipid damage

Running Title: *Cystoseira compressa* extract and chilled horse mackerel quality

INTRODUCTION

Seaweeds are nowadays the raw material for the industrial production of agar, carrageenan and alginates, while they continue to be widely consumed as food in Asian countries. All over the world, marine algae are receiving increasing attention as a new source of bioactive compounds (i.e., polyphenols, alkaloids, terpenes, phycocyanins, carotenoids and various enzymes) [1-3] in addition to being a source of beneficial nutrients, such as vitamins, trace minerals, lipids, amino acids, and dietary fibres [4-6]. Among bioactive compounds, polyphenols derived from brown algae, especially phlorotannins, are well known for possessing multiple physiological activities derived from their antioxidant, anticarcinogenic, antimicrobial and anti-inflammatory properties [7-9].

In most cases, alcoholic solvents have been the most preferred for the extraction of bioactive compounds from marine algae [10-12]. However, different extraction methods have shown a marked influence on the yield of different preserving compounds [13-14]. Indeed, water extracts of different algae have led to higher yields than extracts prepared with other solvent types [15-16], so that higher contents of relatively polar compounds (namely, sulphate polysaccharide, proteins, peptides, glycosides, low-molecular organic acids and salts) would be extracted [17-18]. Indeed, a higher inhibition of microbial activity and lipid oxidation development could be observed in hake (*Merluccius merluccius*) muscle when submitted to a combined ethanol-water extract of *Fucus spiralis* [19].

Algae species belonging to the *Cystoseira* genus have been recognised as one of the most promising algae phylum due their remarkable content in polyunsaturated fatty acids (PUFA) commonly detected in several species when compared to other algal phyla [20-21]. Several *in vitro* studies reported the preservative effect of certain bioactive compounds present in such algae. Thus, the antimicrobial effect of methanol extracts from *Cystoseira hakodatensis* [22] and the antioxidant effect of *Cystoseira barbata* [16], *Cystoseira tamarisfolia* [8] and *C. hakodatensis* [5] extracts have been reported. Andrade et al. [23] pointed out the potential interest of *C. tamarisfolia*, *Cystoseira nodicaulis* and *Cystoseira usneoides* as food additives due to their potential antiradical activity. Among the potential active compounds, phloroglucinol, manitol, fatty acids, and fucosterol were identified in such study.

Among *Cystoseira* species, *Cystoseira compressa* is an abundant species widely distributed in the northeast Atlantic Ocean (from the South of Spain to the Canary Islands), the Mediterranean Sea and the Black Sea [24]. Previous research has shown a profitable fatty acid composition [25]. Thus, a higher PUFA content (45.9 %) as compared to saturated (34.3 %) and monounsaturated (19.8 %) fatty acids was

determined, being C16:0, C20:4 ω 6 and C18:1 ω 9 the most abundant fatty acids. Additionally, this alga species has shown a low ω 6/ ω 3 fatty acids ratio and interesting atherogenicity and thrombogenic activities [25]. The present study is a first attempt to employ this alga species for the preservation of chilled fish. For it, a combined ethanol-aqueous extract was included in the icing system employed as chilling medium during the storage of horse mackerel (*Trachurus trachurus*) during 11 days. The effects of the alga extract on microbial activity and lipid oxidation development were evaluated.

MATERIALS AND METHODS

Preparation of *C. compressa* extracts and icing systems

Fresh *C. compressa* specimens were collected at the Oran coast, Western Algeria (35°44'29.72''N and 0°50'14.21''W). Alga samples were rinsed up with tap water to remove salts, sand and epiphytes and dried at room temperature (23 ± 2 °C) for 72 h under the dark. The dried material was milled, powdered and stored at -20 °C until used for further experiments. Species identification was carried out by employing the *Algabase* site (www.algabase.com) [26].

To prepare the combined alga extract, 20 g of dried alga were mixed with absolute ethanol (60 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 °C. This procedure was carried out two more times, being all the collected extracts pooled together and adjusted to 200 mL with absolute ethanol. Then, the remaining lyophilised alga was mixed with distilled water (60 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 °C. This procedure was carried out two more times, being all the collected extracts pooled together and adjusted to 200 mL with distilled water.

Two different concentrations of the combined alga extract were considered, so that two different kinds of ice were prepared. For the most concentrated, 180 mL of the ethanol extract and 180 mL of the aqueous extract were pooled together and adjusted to 6 L with distilled water. This solution was placed in a polyethylene bag, stored frozen at -18 °C and later used as icing medium (alga high content, AHC batch). To prepare the less concentrated batch, 20 mL of the ethanol extract and 20 mL of the aqueous extract were pooled together and adjusted to 6 L with distilled water. Previously, 160 mL of absolute ethanol were also included in this solution, so that the same content of ethanol was present in both icing media. The resulting solution was also placed in a polyethylene bag, stored frozen at -18 °C and later used as icing medium (alga low content, ALC batch).

To prepare the ice employed as Control batch, 180 mL of absolute ethanol were diluted to 6 L with distilled water. The solution was packaged and kept frozen in the same way as the other two solutions. Before addition to fish specimens, the different kinds of ices were ground to obtain ice flakes. Experimental conditions (namely, content of dried 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 and aqueous extracts from other algae species on lean fish (hake, *Merluccius merluccius*; megrim, *Lepidorhombus whiffiagonis*) [19, 27].

Fish material, processing and sampling

Fresh horse mackerel (60 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 specimens were kept in ice. The length and weight of the fish individuals ranged from 21 to 24 cm and from 170 to 190 g, respectively.

Upon arrival to the laboratory, six specimens were separated and analysed as initial fish (day 0). These specimens were divided into three groups (two specimens per group) that were analysed independently (n=3). The remaining fish specimens were divided into three batches (18 specimens in each batch), that were placed in different boxes and directly surrounded by different kinds of ice (Control, ALC and AHC batches, respectively), prepared as previously described. All batches were placed inside a refrigerated (2 ± 1 °C) room. Boxes allowing draining of melted ice were employed during storage. In all batches, ice was renewed when necessary to maintain the 1:1 fish:ice ratio. Fish specimens from all batches were stored for 11 days, samples being taken for analysis on days 4, 7 and 11. At each sampling time, six specimens 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

Horse mackerel 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 [28-29]. A 0.1 % peptone-water solution was employed to prepare serial dilutions from the different microbial extracts.

Total aerobes were investigated on plate count agar (PCA, Oxoid Ltd., London, UK) after incubation at 30 °C for 48 h. Psychrotrophes were 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 incubation for 24 h at 37 ± 0.5 °C. Microorganisms showing a proteolytic or lipolytic phenotype were analysed on casein-agar medium and tributyrine-agar, respectively, after incubation at 30 °C for 48 h, according to previous research [30].

For all kinds of analyses, microbial counts 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 horse mackerel quality were carried out in triplicate on fish white muscle. Solvents and chemical reagents employed were of reagent grade in all cases (Merck, Darmstadt, Germany).

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

Trimethylamine-nitrogen (TMA-N) values were determined using the picrate colorimetric (Beckman Coulter, DU 640; London, UK) method, as previously described by Tozawa et al. [31]. This method involved the preparation of a 5 % trichloroacetic acid extract of fish muscle (10 g/25 mL). The results are expressed as mg TMA-N kg⁻¹ muscle.

Lipids were extracted from the fish muscle by following the Bligh and Dyer [32] method. Evolution of the free fatty acid (FFA) content was determined in the lipid extract of the horse mackerel muscle in agreement with the Lowry and Tinsley [33] method, which is based on a complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) determination. Results are depicted as mg FFA kg⁻¹ muscle.

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 [34]. Results were calculated as meq active oxygen kg⁻¹ lipids.

The thiobarbituric acid index (TBA-i) was investigated in agreement with the Vyncke [35] procedure, based on the reaction between thiobarbituric acid and an aliquot of a trichloroacetic acid extract of the fish 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 horse mackerel 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 fish muscle [36]. 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 microbiological and chemical analyses were subjected to the one-way analysis of variance to explore differences resulting from the effect of the icing condition; comparison of means was carried out using the least-squares difference (LSD) procedure. Analyses were carried out by employing the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA). For all kinds of analyses, differences among batches were considered significant for a confidence interval at the 95 % level ($p < 0.05$).

RESULTS AND DISCUSSION

Quality evaluation by assessment of microbiological analyses

Aerobic mesophiles were determined in all three batches (Figure 1). The two batches including *C. compressa* alga extracts (i.e., ALC and AHC) allowed a better control of this microbial group as compared with the control batch. Thus, significant ($p < 0.05$) differences were determined between both alga batches and control batch on days 7 and 11. Differences between batches reached a maximum of 2 log units on day 11, this revealing 99 % reduction of aerobes in ALC batch as a consequence of the antimicrobial effect of the alga in the icing medium.

With respect to psychrotrophes, the antimicrobial effect of the presence of the *C. compressa* alga extract in the icing medium also provided a protective effect against this microbial group (Figure 2). Thus, both icing media including alga extracts, either at low or high concentration, exhibited

psychotrophes counts significantly ($p<0.05$) lower than the control batch. Differences in the microbial numbers reached a maximum of 1.68 log units on day 11. Interestingly, and as in the case of aerobes (Figure 1), the batch including lower concentration of *C. compressa* alga in the icing medium provided at least the same beneficial effect as the batch including the higher alga concentration, this indicating that the concentrations of the antimicrobial compounds in the ALC batch were sufficient as to provide a significant protection against microbial growth in horse mackerel muscle.

The investigation of Enterobacteriaceae in all three batches showed an inhibitory effect ($p<0.05$) on this microbial group derived from the presence of both *C. compressa* alga extracts in the icing medium at day 4 (Table 1). After that time, differences among batches could not be considered as significant ($p>0.05$); however, the growth of Enterobacteriaceae in the ALC batch and to a lesser extension in the AHC batch, was slowed down by the presence of the *C. compressa* alga extract in the refrigeration medium. Numbers of this microbial group were in all cases below 4 log units in control and AHC batches and even lower, below 3 log units, in the ALC batch. Accordingly, Enterobacteriaceae levels can be considered as low in all cases.

Table 1 summarises the results of the investigation of specific spoilage microorganisms (SSM) exhibiting proteolytic or lipolytic phenotypes in horse mackerel muscle. Interestingly, and as in the case of other microbial groups considered in this study, the presence of the *C. compressa* alga extracts in the icing media provided a protective effect with respect to the development and growth of SSM. In this sense, bacteria exhibiting a proteolytic phenotype were partially inhibited in ALC and AHC batches, as compared to the control batch. Thus, significant ($p<0.05$) differences between batches including or not alga extracts were determined at all sampling times (4-11 days). Such differences reached a maximum of 1.47 log units for proteolytic bacteria on day 11.

With respect to lipolytic bacteria, this is a major concern in the case of medium fatty-fish species such as horse mackerel. The inclusion of *C. compressa* alga extracts in the icing media, AHC and ALC batches, also led to a lower average growth of this group of SSM as compared to the control batch. Differences were found to be significant ($p<0.05$) on day 7 for both alga batches and throughout the whole storage period for the ALC batch. The differences between ALC and control batches reached a maximum of 1.66 log units on day 7. The results compiled in Table 1 allowed concluding that the expected microbial damage caused to horse mackerel muscle by microbial extracellular proteases and

lipases may be at least partially limited by the inclusion of antimicrobial compounds provided by the alga extract.

Although a wide variety of information related to the *in vitro* antibacterial activity of brown algae has been published, previous research concerning their practical application for the preservation of seafood can be considered scarce. In the present study, *C. cystoseira* extracts were included for the first time in the icing media for the chilled storage of a marine fish species. As a result, combined extracts of this alga were found to exert a remarkable antimicrobial effect on aerobes, psychrotrophes and proteolytic bacteria, while some inhibitory effect on lipolytic and Enterobacteriaceae could also be observed. The melting of the ice crystals containing the alga extracts might exert both washing and inhibitory effects that would reduce the microbial load on the fish surface, this limiting the microbial diffusion towards horse mackerel muscle. Remarkably, scarce differences were observed among individuals corresponding to both alga batches.

The presence of natural components with antimicrobial activity is widespread in macroalgae and a wide range of metabolites have been isolated and characterised. In the Order Fucales (Class Phaeophyceae), natural products of mixed biosynthesis, consisting of terpenes, polyphenols, oligomeric phlorotannins, hydroquinones and halogenated alkanes and alkenes have been identified as key antimicrobial components [9, 37]. 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 [4, 7].

Concerning *Cystoseira* genus, scarce applications on seafood preservation have been reported. Thus, a methanol extract of *C. hakodatensis* exhibited inhibitory effect on the growth of *Bacillus cereus* and *Bacillus licheniformis* *in vitro*, and the major component involved in antimicrobial activity was successfully isolated from the hydrophobic fraction of the extract [22]. Furthermore, Andrade et al. [23] proved that *C. tamarisfolia*, *C. nodicaulis* and *C. usneoides* were among the most active algae species against enzyme activity with biological significance. Such effect was explained on the basis of their high content of phloroglucinol, mannitol, fucosterol and oleic, arachidonic and eicosapentaenoic acids. Also related to other brown seaweeds, the inclusion in the icing medium of a combined ethanol-aqueous extract of *Fucus spiralis* led to a marked inhibition of microbial activity in hake (*Merluccius merluccius*) muscle during chilled storage [19]. The inhibition of microbial activity was also observed in chilled

megrim (*Lepidorhombus whiffiagonis*) muscle when ice including ethanolic extracts of *Bifurcaria bifurcata* was employed [27].

Chemical assessment of microbial activity

A progressive increase of pH value with chilling time was observed, this being in agreement with the evolution of microbial degradation mechanisms (Table 2). Comparison among batches revealed scarce significant differences ($p < 0.05$). However and in agreement with the above-mentioned results on microbial counts, higher average values were determined throughout the whole experiment in samples corresponding to the Control batch. Consequently, a slight inhibitory effect on pH increase was derived from the presence of *C. compressa* extract in the icing medium.

Increases in the pH value of fish muscle indicate the accumulation of alkaline compounds, such as ammonia, trimethylamine and other nitrogenous compounds, which mainly derive from microbial activity [7, 38]. Previous studies have reported a slower pH increase as a result of the inclusion of a *B. bifurcata* ethanolic extract in the icing system for the storage of chilled megrim (*Lepidorhombus whiffiagonis*) [27].

A progressive increase in TMA concentration was observed in all fish batches as storage time progressed (Table 2). Such increase was found to be lower in fish corresponding to the AHC batch. Comparison among batches showed a significant inhibition ($p < 0.05$) in samples corresponding to both alga batches for the 4-7-day period. At advanced storage periods, only the AHC batch showed inhibitory effect. Comparison among both alga batches showed lower average values in fish muscle corresponding to the AHC batch.

During the storage of fish under refrigeration conditions, TMA is produced as a result of trimethylamine oxide breakdown by microbial enzyme catalysis. TMA values determined in this work were in agreement with the microbial numbers that indicated microbial inhibition by the presence of *C. compressa* in the icing medium. An inhibitory effect on TMA formation was also observed in chilled megrim when employing ice including an ethanolic extract of *B. bifurcata* [27].

Quality evaluation by analysis of lipid damage

FFA concentrations in chilled fish increased with storage time in all batches (Table 2). Such increase was relevant on day 7 for the Control batch and on day 11 for AHC and ALC batches. Comparison among

icing media revealed higher FFA levels throughout storage in horse mackerel corresponding to the Control batch ($p < 0.05$). However, no differences ($p > 0.05$) could be depicted between both batches including the alga extract. Thus, an inhibitory effect on FFA formation derived from the use of both alga concentrations (i.e., ALC and AHC batches) in the icing media could be concluded. This result is also in agreement with the inhibition of lipolytic bacteria described above.

FFA formation can be considered the result of endogenous enzyme and microbial activities [38]. Prior to the end of the microbial lag phase, FFA formation is primarily a result of endogenous enzymes (i.e., lipases and phospholipases) activity. Afterwards, microbial activity becomes more relevant due to bacterial catabolic processes. In the present study, the relevant higher values found in the Control batch during the 7-11-day period would correspond to microbial activity. In such a period, a marked inhibition of FFA formation was observed in fish batches including *C. compressa* extracts. However, no differences were found between AHC and ALC batches. The inhibition of FFA formation had also been observed in chilled megrim when employing an ethanolic extract of *B. bifurcate* [27] as well as an ethanolic-aqueous extract of *F. spiralis* [19].

With some exceptions, a slight increase of peroxides content throughout storage could be observed in all batches (Table 3). However, PV were in all cases low (< 2.94), so that primary lipid oxidation cannot be considered an important spoilage mechanism in the present study. No significant differences ($p > 0.05$) could be depicted among batches, so that no effect of the presence of the alga in the chilling medium on peroxides formation could be concluded.

TBARS formation increased in all batches on the 0-7-day period (Table 3). After that time, only fish specimens corresponding to the AHC batch exhibited a further increase of such oxidised compounds. Although certain significant differences ($p < 0.05$) among batches could be observed, such comparison did not provide a general trend about the effect of the alga presence in the chilling medium. At the end of the storage period, the highest average value was determined in the AHC batch, while the lowest corresponded to the ALC batch.

A progressive formation of tertiary lipid oxidation compounds (i.e., fluorescence ratio) along storage time was determined in all batches (Table 3). Comparison among batches did not provide significant differences ($p > 0.05$). However, fish specimens corresponding to the Control batch exhibited the highest average FR values at all sampling times, so that a partial inhibitory effect of the alga extracts on tertiary lipid oxidation can be concluded.

Partial lipid oxidation inhibition observed in the current study can be explained on the basis of that brown macroalgae produce a wide range of antioxidant compounds, these including polyphenols, phycocyanins and carotenoids [3, 12]. Among them, phlorotannins and polyphenols formed by oligomers and polymers of phloroglucinol (1,3,5-trihydroxy benzene) have been reported to be the largest group of seaweed phenolic compounds [10, 13, 37]. Furthermore, fucoxanthin has been identified as the dominant carotenoid in brown seaweeds [1].

Previous reports have referred to *in vitro* experiments related to the antioxidant effect of different algae species belonging to the *Cystoseira* genus. Thus, *C. tamarisfolia* exhibited strong chelator activity that was attributed to the presence of phlorotannins in methanol extracts [11]. Furthermore, ethanol extracts of *C. tamarisfolia*, *C. nodicaulis* and *C. usneoides* showed a strong inhibition of free radicals formation, which was explained on the basis of their high contents on phloroglucinol, mannitol, oleic, fucosterol and arachidonic and eicosapentaenoic acids [23]. A strong antioxidant activity has also been reported for *C. tamarisfolia* [8] and *C. hakodatensis* extract [5]. Such activity was linked not only to the high content of phenolic compounds, but also to the presence of fucoxanthin. Aqueous and ethanol extracts of *C. barbata* were included in a tuna-skin gelatine film [16]; as a result, alga extracts provided to the film an increased ferric-reducing ability and a scavenging effect on DPPH free radical formation.

With respect to other brown seaweed, a combined ethanol and aqueous extract of *F. spiralis* exerted inhibitory effect on lipid oxidation (i.e., fluorescent compounds formation) in chilled hake when included in the icing medium [19]. Likewise, inhibition of lipid oxidation (i.e., fluorescent compounds formation) was also observed in chilled megrim when an ethanolic extract of *B. bifurcata* was included in the icing system [27]. Finally, a 50 % aqueous-ethanol extract of the red alga *Polysiphonia fucoides* was found to retard lipid oxidation in a food model system consisting of minced Atlantic mackerel (*Scomber scombrus*) [14].

CONCLUSIONS

A combined ethanolic and aqueous extract of *C. compressa* was included in the icing medium employed for the chilled storage of a fatty fish species. On the basis of the microbial counts assessment (aerobes, psychrotrophs, proteolytic, lipolytic and Enterobacteriaceae bacteria) and the determination of chemical parameters related to microbial activity (pH and TMA), a relevant inhibition of the microbial activity development in chilled horse mackerel could be concluded. Additionally, a reduction of lipid hydrolysis

(FFA formation) and oxidation (fluorescent compounds formation) events was also observed in batches including *C. compressa* extracts. Interestingly, only slight differences could be observed between fish batches corresponding to the two algae concentrations tested in the current study, this indicating that the concentrations of preservative compounds in the ALC batch were sufficient as to provide a significant protection of horse mackerel muscle.

The novelty of this study lays on the fact that most studies focused on the protective effect of algae have been performed *in vitro*. Accordingly, studies about the application of algae extracts on commercial seafood can be considered very scarce. Interestingly, the icing medium proposed in this work (i.e., a combined ethanolic and aqueous extract) may constitute a promising strategy to open the way to the application of natural algae extracts for improving of quality retention of all kinds of fish species. Such combined extract would provide a wider range of preserving molecules (namely, lipophilic and water-soluble), thus allowing a more complete preservative effect.

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FIGURE LEGENDS

Figure 1: Aerobic mesophile bacteria (log CFU g⁻¹ muscle) assessment* in chilled horse mackerel 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: ALC and AHC (alga low content and alga high content batches, respectively).

Figure 2: Psychrotrophic bacteria (log CFU g⁻¹ muscle) assessment* in chilled horse mackerel 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 employed for icing batches as expressed in Figure 1.

TABLE 1

Microbiological counts assessment (log CFU g⁻¹ muscle)* in chilled horse mackerel muscle stored under different icing conditions**

Chilling storage time (days)	Enterobacteriaceae			Proteolytics			Lipolytics		
	Control	ALC	AHC	Control	ALC	AHC	Control	ALC	AHC
0		1.07 (0.12)			2.69 (0.27)			2.26 (0.24)	
4	2.74 b (0.23)	1.70 a (0.17)	1.78 a (0.16)	5.76 b (0.25)	5.09 a (0.23)	5.05 a (0.37)	5.04 b (0.39)	4.22 a (0.39)	4.28 ab (0.54)
7	2.13 a (0.31)	1.56 a (0.31)	1.63 a (0.35)	6.51 b (0.18)	5.13 a (0.18)	5.53 a (0.50)	6.02 b (0.17)	4.36 a (0.39)	4.43 a (0.23)
11	3.88 a (0.89)	2.92 a (0.60)	3.09 a (1.21)	8.09 b (0.36)	6.95 a (0.42)	6.62 a (0.50)	6.73 b (0.09)	5.20 a (0.35)	6.51 ab (0.94)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each microbial group and for each chilling time, mean values followed by different letters (a, b) indicate significant (p<0.05) differences as a result of the icing condition.

** Abbreviations of icing conditions: ALC and AHC (alga low content and alga high content batches, respectively).

TABLE 2

Assessment of pH, trimethylamine-nitrogen and free fatty acids values* in chilled horse mackerel muscle stored under different icing conditions**

Chilling storage time (days)	pH			Trimethylamine-nitrogen			Free fatty acids		
	Control	ALC	AHC	Control	ALC	AHC	Control	ALC	AHC
0		5.99 (0.02)			0.23 (0.03)			5.43 (1.08)	
4	6.26 b (0.10)	6.03 a (0.08)	6.06 ab (0.16)	3.99 b (0.69)	1.21 a (1.12)	0.80 a (0.44)	5.30 b (1.43)	3.68 a (0.14)	3.27 a (0.50)
7	6.44 a (0.20)	6.16 a (0.29)	6.31 a (0.13)	11.92 b (3.16)	6.28 a (4.71)	5.31 a (1.41)	26.1 b (6.12)	3.00 a (0.43)	3.80 a (0.59)
11	6.43 a (0.13)	6.33 a (0.09)	6.23 a (0.17)	36.68 b (3.49)	38.25 b (16.94)	18.12 a (0.92)	32.25 b (13.00)	8.64 a (2.28)	6.60 a (1.80)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each quality index and for each chilling time, mean values followed 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 3**Assessment of lipid oxidation development* in chilled horse mackerel muscle stored under different icing conditions****

Chilling storage time (days)	Peroxide value			Thiobarbituric acid index			Fluorescence ratio		
	Control	ALC	AHC	Control	ALC	AHC	Control	ALC	AHC
0		0.94 (0.62)			0.19 (0.08)			0.47 (0.07)	
4	1.26 a (0.52)	2.24 a (0.87)	1.64 a (0.50)	0.83 a (0.16)	1.16 b (0.22)	0.97 ab (0.16)	0.68 ab (0.17)	0.67 b (0.13)	0.47 a (0.07)
7	2.92 a (1.69)	0.99 a (0.84)	1.81 a (0.97)	1.84 b (0.28)	1.59 ab (0.41)	1.40 a (0.28)	0.84 b (0.13)	0.51 a (0.10)	0.71 b (0.03)
11	1.49 a (0.83)	2.93 a (1.93)	2.64 a (0.57)	1.82 ab (0.11)	1.74 a (0.16)	2.14 b (0.23)	1.35 a (0.46)	1.27 a (0.19)	0.96 a (0.16)

* 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 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

Figure 1