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Antioxidant and antimicrobial behaviour of alga *Gracilaria gracilis* extracts during hake (*Merluccius merluccius*) chilled storage

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The present research is a first attempt for the use of alga *Gracilaria gracilis* as a source of preservative compounds to be applied during the chilled storage of fish. For it, a combination of ethanolic and aqueous extracts of this edible seaweed were included in the icing medium employed for the chilling storage of hake (*Merluccius merluccius*). Chemical and sensory analyses related to quality loss were carried out in fish throughout 9-day storage. Three different alga concentrations were tested and compared to an icing control (traditional ice). As a result, an inhibitory effect ($p<0.05$) on lipid oxidation development (tertiary oxidation compounds) and microbial activity (trimethylamine formation) was observed in fish corresponding to batches including the two most concentrated alga conditions. However, a definite effect on lipid hydrolysis development (free fatty acids formation) could not be implied ($p>0.05$). Concerning sensory analysis, samples from the same two batches revealed a higher acceptance ($p<0.05$) than control; this difference was based on the evaluation of various descriptors such as skin, external odour, raw flesh odour and cooked flesh odour. On the basis of the presence of antioxidant and antimicrobial molecules, a profitable effect on quality retention of chilled hake is concluded by including *G. gracilis* extracts in the icing system.

Keywords: *Gracilaria gracilis*; *Merluccius merluccius*; Chilling; Antioxidant; Antimicrobial; Shelf life

INTRODUCTION

Marine species commercialised in the fresh state represent the highest proportion in seafood production and human consumption. However, aquatic food products deteriorate rapidly after the death of the animals they derive from due to the effects of a variety of biochemical and microbial degradation mechanisms [1]. In agreement with nowadays consumer demand for high-quality fresh products, food technologists and fish trade have focussed on the search for new and advanced strategies to maintain the original quality of marine species [2]. Among them, the inclusion of natural preservative compounds in the icing system employed for the chilling storage has been developed. Thus, previous research accounts for the employment of vegetable extracts [3, 4], low-molecular weight organic acids [5, 6] and edible seaweed [7].

Gracilaria gracilis is a red alga (Rhodophyta) widely distributed in different parts of the world. Most previous research on this species has been focused on its employment as a source of agar [8, 9] and as indicator of contamination [10, 11]. However, research focused on its chemical composition has shown that this species can be considered as a multi products source for biotechnological, nutraceutical and pharmaceutical applications [12-14] in agreement with its high contents of total polyphenolic compounds, microelements, polysaccharides, etc.; additionally, a high antioxidant and radical scavenging activity was proved [12, 13].

The present research is a first attempt for the use of *G. gracilis* as a source of preservative compounds to be applied during the chilled storage of fish. For it, ethanolic and aqueous extracts of this edible seaweed were included in the icing medium employed for the chilling storage of hake (*Merluccius merluccius*). Chemical and sensory properties related to quality loss were analysed in fish throughout 9-day storage.

MATERIALS AND METHODS

Preparation of G. gracilis extracts and icing systems

The lyophilised alga *G. gracilis* was provided by Porto-Muiños (Cerceda, A Coruña, Spain). Three different concentrations were tested in the present study. For the lowest one, 1 g alga was mixed with absolute ethanol (2 × 120 mL), stirred for 30 s and

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centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the supernatant was recovered and filled to 80 mL with absolute ethanol. Additionally, the remaining lyophilised alga was mixed with distilled water (2 × 120 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the supernatants were recovered, pooled together with the previously obtained ethanolic extract and the mixture was diluted to 6 L with distilled water (0.17 g lyophilised alga L^{-1} aqueous solution). This solution was packaged in polyethylene bags, kept frozen at –18 ºC and later used as icing medium (G-1 condition).

Likewise, 4 and 15 g of lyophilised alga were extracted successively with ethanol and water, and finally diluted to 6 L to obtain 0.67 and 2.50 g lyophilised alga L^{-1} aqueous solutions, respectively. These solutions were also packaged, kept frozen and further employed as G-2 and G-3 icing conditions, respectively. Besides, 80 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 and further employed as Control batch (G-0 condition). Before addition to individual fish specimens, the different icing systems were ground to obtain ice flakes.

Fish material, processing and sampling

Fresh European hake (*Merluccius merluccius*) (78 individuals) were caught near the Galician Atlantic coast (north-western Spain) and transported on ice to the laboratory ten hours after catching. The length and weight of the fish specimens were in the following ranges: 30 to 34 cm and 160 to 200 g, respectively.

Upon arrival in the laboratory, six individual fishes were separated and analysed as starting raw fish (day 0); for it, three different groups (two individuals per group) were analysed independently to perform statistical analysis (n=3). The remaining fish were divided into four batches (18 individuals in each batch), placed in boxes and directly surrounded by the four kinds of ices previously mentioned (G-0, G-1, G-2 and G-3 conditions), respectively; a 1:1 fish-to-ice ratio was employed. All batches were placed in a refrigerated room (4 ºC). Boxes employed allowed draining and ice was renewed when required. Fish samples from all batches were taken for analysis on days 2, 6 and 9. At each sampling time, six individuals of each batch were taken for analysis, being considered into three groups (two individuals in each group) that were studied independently (n=3).

Sensory analysis was carried out on the whole fish; chemical analyses were carried out on the white muscle.

Chemical analyses

All solvents and chemical reagents used were of reagent grade (Merck, Darmstadt, Germany).

Total polyphenols content of lyophilised alga was assessed by means of the Folin-Ciocalteu colorimetric method (Cary 3E UV–Visible spectrophotometer, Varian; Mulgrave, Victoria, Australia) as described previously [15]. Measurements were made in triplicate. Gallic acid (GA) was used as standard. Results were expressed as mg GAE g^{-1} lyophilised alga.

Lipids were extracted from the fish white muscle by the Bligh and Dyer [16] method, which employs a single-phase solubilisation of the lipids using a chloroform-methanol (1:1) mixture. The results were calculated as g lipid kg^{-1} muscle.

Free fatty acid (FFA) content was determined in the lipid extract of the fish muscle by the Lowry and Tinsley [17] method based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment. Results were calculated as mg oleic acid kg^{-1} muscle and expressed as mg FFA kg^{-1} muscle.

The peroxide value (PV) was determined spectrophotometrically (Beckman Coulter, DU 640; London, UK) using the lipid extract *via* previous peroxide reduction with ferric thiocyanate according to the Chapman and McKay [18] method. The results were expressed as meq active oxygen kg^{-1} lipids.

The thiobarbituric acid index (TBA-i) was determined according to Vyncke [19]. This method is based on the reaction between a trichloroacetic acid extract of the fish muscle and thiobarbituric acid. Content of thiobarbituric acid reactive substances (TBARS) was spectrophotometrically measured at 532 nm and calculated from a standard curve using 1,1,3,3-tetraethoxy-propane (TEP); results were expressed as mg malondialdehyde kg^{-1} muscle.

The formation of fluorescent compounds (Fluorimeter LS 45; Perkin Elmer España; Tres Cantos, Madrid, Spain) was determined by measurements at 393/463 nm and 327/415 nm as described by Losada *et al*. [20]. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission maximum and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 μg mL^{-1} in 0.05 M H_2SO_4) at the corresponding wavelength. The 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 resulted from the lipid extraction of the fish muscle [16].

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*. [21]. This method involves the preparation of a 5% trichloroacetic acid extract of fish muscle (10 g/25 mL). The results were expressed as mg TMA -N kg^{-1} muscle.

Sensory analysis

Sensory analysis was conducted by a sensory panel that consisted of five experienced judges who adhered to traditional guidelines concerning fresh and refrigerated fish, which were adapted to hake [22]. Before carrying out the present experiment, the judges received training on refrigerated hake. Special attention was paid to the evolution of the sensory descriptors that were found as limiting factors for the shelf life.

For the sensory acceptance, four categories were ranked [22]: highest quality (E), good quality (A), fair quality (B) and unacceptable quality (C). Sensory assessment of the fish included the following descriptors: skin and mucus development, eyes, external odour, gills appearance and odour, consistency, flesh (raw and cooked) odour and flesh taste. At each sampling time, whole fish specimens were coded with 3-digit random numbers and presented to the panellists in individual trays, which were scored individually.

Statistical analysis

Data obtained from the different chemical analyses were subjected to the ANOVA method to explore differences resulting from the effect of the chilling condition; the comparison of means was performed using the least-squares difference (LSD) method. Data obtained from the sensory evaluation were analysed by the non-parametric Kruskal-Wallis test. In all cases, analyses were carried out using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences between batches were considered significant for a confidence interval at the 95 % level ($p<0.05$) in all cases.

RESULTS AND DISCUSSION

Assessment of quality loss by chemical indices

Lipid content of hake fish ranged between 5.0 and 5.7 g kg^{-1} muscle. Lipid hydrolysis development was measured by the FFA determination (Fig. 1). A marked increase was observed in all kinds of samples at the end of the storage when compared with the initial fish.

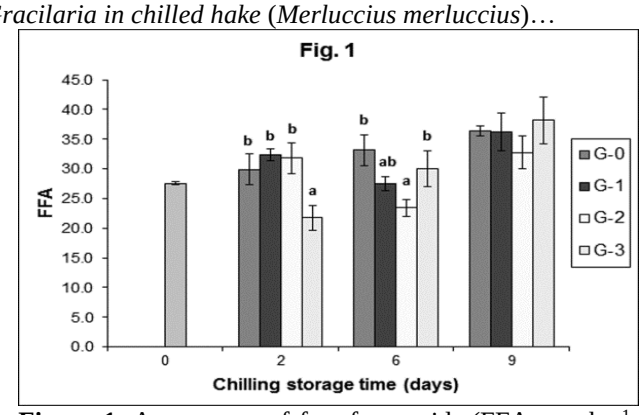

Figure 1. Assessment of free fatty acids (FFA; mg kg^{-1} muscle) formation* in chilled hake muscle stored under various icing conditions**

* Average values of three replicates (*n*=3). Standard deviations are indicated by bars. Average values accompanied by different letters (a, b) denote significant differences ($p<0.05$). No letters are included when significant differences were not found ($p>0.05$).

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

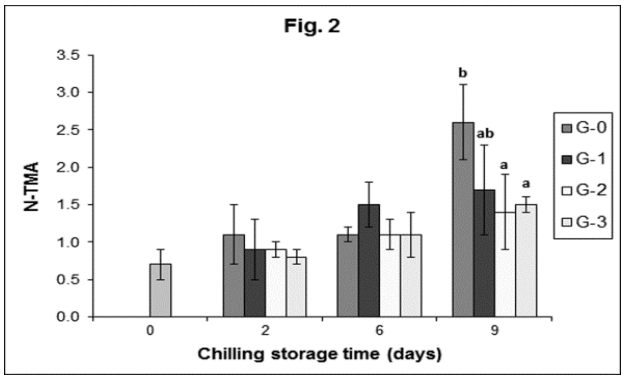

Figure 2. Assessment of trimethylamine-nitrogen (TMA-N; mg kg^{-1} muscle)* in chilled hake muscle stored under various icing conditions**

* Average values of three replicates (*n*=3). Standard deviations are indicated by bars. Average values accompanied by different letters (a, b) denote significant differences ($p<0.05$). No letters are included when significant differences were not found ($p>0.05$).

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

A definite effect of *G. gracilis* extracts in the chilling system on the lipid hydrolysis development could not be proved; however, at day 2, the lowest FFA formation ($p<0.05$) was found in samples corresponding to G-3 condition, while the lowest average values in the 6-9-day period corresponded to the G-2 batch.

Previous related research showed opposite conclusions. In agreement with the present results, the presence of citric, lactic and ascorbic acids in the icing system did not lead to a marked inhibitory effect on FFA formation in chilled horse mackerel [5]; the same conclusion was obtained by Özyurt *et al*. [4] by employing a rosemary extract during the chilling storage of sardine. Contrary, an inhibitory

effect on fish FFA formation could be observed by applying an ethanolic-aqueous extract of *Fucus spiralis* during the chilled storage of hake [7]; a similar result was attained by Quitral *et al*. [3] by employing ice including rosemary or oregano extracts in chilled Chilean jack mackerel.

Lipid oxidation development was measured by different and complementary indices. Primary compounds assessment (PV) (Table 1) showed a low peroxide formation in all kinds of samples; values were in all cases below 3.1. Scarce differences could be observed between samples, so that a definite effect of alga presence in the ice could not be proved ($p>0.05$) for the peroxide content in chilled hake.

Secondary lipid oxidation development was measured by means of the TBA-i (Table 1). This index showed a marked increase in all samples after a 9-day chilled storage. However, values obtained can be considered low in all cases, being all scores lower than 0.22. Consequently, differences between samples can be considered scarce. Interestingly, lower average values were observed in fish corresponding to the highest *G. gracilis* presence (G-3 condition) throughout the whole experiment; however, differences with the control were not found significant ($p>0.05$).

Interaction compounds formation was measured by the FR (Table 1). A progressive formation of such compounds could be observed for all samples throughout the whole chilling storage.

At the end of the experiment, all kinds of samples including *G. gracilis* extracts in the icing system showed a lower FR ($p<0.05$) than the control. Consequently, an inhibitory effect on tertiary lipid oxidation compounds could be concluded.

Table 1. Lipid oxidation development* in chilled hake muscle stored under various icing conditions**

Quality index	Chilling time (days)	Icing condition			
		G-0	G-1	G-2	G-3
Peroxide value (meq active oxygen kg^{-1} lipids)	0	1.84 (0.13)			
	2	2.23 b (0.04)	1.83 ab (0.45)	1.72 a (0.01)	1.81 ab (0.73)
	6	2.96 (0.01)	2.57 (0.37)	2.67 (0.47)	2.57 (0.41)
	9	1.72 ab (0.32)	1.09 a (0.40)	2.49 ab (0.92)	3.00 b (0.28)
Thiobarbituric acid index (mg malondialdehyde kg^{-1} muscle)	0	0.09 (0.01)			
	2	0.07 (0.02)	0.12 (0.06)	0.05 (0.04)	0.02 (0.00)
	6	0.06 (0.02)	0.08 (0.01)	0.06 (0.04)	0.03 (0.02)
	9	0.18 ab (0.01)	0.15 a (0.03)	0.22 b (0.01)	0.12 a (0.05)
Fluorescence ratio	0	2.72 (0.65)			
	2	2.61 b (0.09)	2.19 ab (0.66)	2.02 a (0.19)	2.26 ab (0.53)
	6	3.31 (0.77)	3.02 (0.11)	3.03 (0.15)	2.98 (1.18)
	9	4.18 b (0.15)	3.24 a (0.16)	3.18 a (0.50)	3.11 a (0.23)

* Average values of three replicates (n=3); standard deviations are indicated in brackets. Average values followed by different letters (a, b) denote significant ($p<0.05$) differences as a result of the icing medium. No letters are indicated when differences were not found ($p>0.05$). ** Icing conditions: G-1, G-2 and G-3 (ices prepared from 0.17, 0.67 and 2.50 g L^{-1} aqueous solution of *G. gracilis* extract, respectively); G-0 (Control; without alga extract in the icing system).

This result can be explained on the basis that previous research on *G. gracilis* has shown a marked radical-scavenging ability and antioxidant behaviour [12, 13]. Interestingly, a polyphenol content of 3.1±0.8 mg GAE g^{-1} lyophilised alga was obtained in the current study. This polyphenol

content can be considered very similar to the one described by Heffernan *et al*. [13] (2.79-5.36 mg GAE g^{-1}) but lower than the one reported by Francavilla *et al*. [12] (2.3-65.0 mg GAE g^{-1}).

In a closely related research [7], the inclusion in the icing medium of alga *F. spiralis* led to a lower FR in hake muscle during the chilled storage. Previous research related to other natural sources of antioxidant compounds has shown a preservative effect when present in the icing medium.

This accounts for Chilean jack mackerel as a result of including an oregano or rosemary extract in the icing system [3], horse mackerel by the presence of an organic acid mixture (citric, lactic and ascorbic acids) as ice system [5] and sardine by including a rosemary extract in the chilling medium [4].

Table 2. Sensory acceptance* of chilled hake stored under various icing conditions**

Descriptor	Chilling time (days)	Icing condition			
		G-0	G-1	G-2	G-3
Skin	0		E		
	2		A		
	6		A		
	9	C^y		B^z	
Eyes	0		E		
	2		A		
	6		A		
	9		B		
External odour	0		E		
	2		A		
	6	B^y		A^z	
	9	C^y		B^z	
Gills	0		E		
	2		A		
	6		B		
	9		B		
Consistency	0		E		
	2		A		
	6		B		
	9		B		
Raw flesh odour	0		E		
	2		A		
	6		A		
	9	C^y		B^z	
Cooked flesh odour	0		E		
	2		A		
	6		A		
	9	C^y		B^z	
Flesh taste	0		E		
	2		A		
	6		A		
	9		B		

* Scores as expressed in the material and methods section. For each descriptor, scores with different superscripts (z, y) indicate significant ($p<0.05$) differences as a result of the icing condition. No superscripts are included when differences were not found ($p>0.05$).

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

Microbial activity development was measured by the TMA detection (Fig. 2). Thus, a progressive formation in all kinds of samples with chilling was obtained for this quality parameter. Comparison between samples showed lower values ($p<0.05$) in samples corresponding to G-2 and G-3 batches

when compared with the control. Accordingly, an inhibitory effect on TMA formation was implied by the presence of *G. gracilis* in the icing system. Interestingly, none of the samples surpassed the legal limit established for this species (5 mg kg^{-1}) [23].

Volatile amine compounds such as TMA have been reported to be produced mostly as a result of microbial development during fish chilled storage [1]. The inhibitory effect of *G. gracilis* on microbial activity obtained in the present study can be explained on the basis of previous research pointing out the possibility of using this alga species as a multi-product source for nutraceutical and pharmacological application [12, 14]. The antimicrobial effect of algae in general has been attributed especially to the presence of terpenes, polyphenols and oligomeric phlorotannins [24, 25].

Previous research shows the inhibitory effect on TMA content as a result of including a wide range of natural preservative compounds in the icing system. Thus, the presence of citric, lactic and ascorbic acids led to a lower TMA formation in chilled mackerel [6]. Microbial activity inhibition was also detected by Özyurt *et al*. [4] by the presence of rosemary extract in the ice during sardine chilling; thus, a lower formation of histamine and putrescine was detected in fish samples corresponding to batches including the plant extract. Contents on microbial counts of various bacteria groups showed to decrease as a result of including wild-thyme hydrosol in the icing medium during Transcaucasian barb chilled storage [26]. Microbial activity inhibition was also observed in previous related research [7]; thus, lower counts of aerobe, psychrotroph, proteolytic and lipolytic bacteria were implied in chilled hake by employing ice including ethanolic-aqueous extracts of alga *F. spiralis*.

Assessment of sensory acceptance

Sensory acceptance was evaluated by analysis of different descriptors. Results obtained are expressed in Table 2. For all kinds of samples, all descriptors showed a sensory quality decrease with chilling time. Fish corresponding to control and G-1 batches were found rejectable at the end of the experiment. Comparison among icing conditions showed higher scores ($p<0.05$) for fish corresponding to G-2 and G-3 batches when compared with their counterparts from G-1 and G-0; such differences were obtained for skin, external odour, raw flesh odour and cooked flesh odour. Consequently, a preservative effect was concluded by the presence of *G. gracilis* extracts in the icing medium. This effect is in agreement with the above-mentioned results on antioxidant and antimicrobial properties found in the current alga extracts.

An increased shelf life time has also been observed as a result of including preservative compounds in the icing system. This is the case of a rosemary extract during chilled storage of sardine [4], an acid mixture (citric, lactic and ascorbic) during the chilled storage of horse mackerel [5] and wild-thyme hydrosol in chilled transcaucasian barb [26].

CONCLUSIONS

G. gracilis extracts were tested as a source of natural preservative compounds to be included in the icing system employed during the chilled storage of hake. As a result, an inhibitory effect on lipid oxidation development (tertiary oxidation compounds) and microbial activity (trimethylamine formation) was observed in fish corresponding to batches including the two most concentrated alga conditions. However, a definite effect on lipid hydrolysis development could not be implied ($p>0.05$). Concerning sensory analysis, samples from the same two batches revealed a higher acceptance ($p<0.05$) than control; this difference was based on the evaluation of various descriptors such as skin, external odour, raw flesh odour and cooked flesh odour. On the basis of the presence of antioxidant and antimicrobial compounds, a profitable effect on quality retention of chilled hake is achieved by including *G. gracilis* extracts in the icing system.

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АНТИОКСИДАНТНИ И АНТИМИКРОБНИ СВОЙСТВА НА ЕКСТРАКТИ ОТ ВОДОРАСЛОТО *Gracilaria gracilis* ПРИ СЪХРАНЕНИЕ НА ХЕК (*Merluccius merluccius*) В ОХЛАДЕНО СЪСТОЯНИЕ

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(Резюме)

Настоящото изследване е първи опит за използване на водораслото *Gracilaria gracilis* като източник на консервиращи съединения, които да се използват при съхранение на риба в охладено състояние. За целта, комбинация от етанолов и воден екстракт от ядивното морско водорасло е добавена към замразяващата среда, използвана за съхраняване на хек (*Merluccius merluccius*) в охладено състояние. За установяване на евентуално влошаване на качеството са проведени химически и сензорни анализи на рибата в продължение на 9-дневно съхранение. Изследвани са три концентрации на водораслото и са сравнени с контролна проба (традиционно охлаждане с лед). Установен е инхибиторен ефект ($p<0.05$) върху окислението на липидите (образуване на третични окислени съединения) и микробната активност (образуване на триметиламин) в сериите от риба, съдържащи двете най-високи концентрации от водораслото. Не е установен обаче ефект ($p>0.05$) върху хидролизата на липидите (образуване на свободни мастни киселини). Сензорният анализ на проби от същите две серии показва по-добро отнасяне ($p<0.05$) в сравнение с контролата. Разликата се основава на оценката на различни дескриптори като кожа, външен мирис, мирис на суровото месо и мирис на свареното месо. Направен е изводът, че включването на екстракт от *G. gracilis* в охладителната система има положителен ефект върху запазването на качеството на охладения хек като следствие от присъствието на антиоксидантни и антимикробни молекули.