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Quality increase of refrigerated fish by employment of a gelatine biofilm including a protein hydrolysate obtained from alga *Fucus spiralis*

D. D. Castro-Enríquez[1], J. M. Miranda[2], J. Barros-Velázquez[2], S. P. Aubourg[3*]

[1] *Departamento de Investigación y Posgrado en Alimentos, University of Sonora, Hermosillo, Sonora, Mexico*
[2] *Departamento de Química Analítica, Nutrición y Ciencia de los Alimentos, Facultad de Ciencias Veterinarias, Universidad de Santiago de Compostela, Lugo, Spain*
[3] *Departamento de Ciencia y Tecnología de Alimentos, Instituto de Investigaciones Marinas (CSIC), Vigo, Spain*

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This study focusses on the employment of a gelatine-based biofilm during fish refrigeration. In it, a *Fucus spiralis* protein hydrolysate (PH) is included in the biofilm and used as packaging system in European hake (*Merluccius merluccius*) slices during the refrigerated storage (up to 6 days at 4 ºC). A progressive quality loss can be observed in hake slices by increasing the storage time. However, comparison with control fish shows a partial inhibitory effect on microbial activity development (counts assessment of aerobes, psychrotrophs, *Enterobacteriaceae*, proteolytics, lipolytics and anaerobes) and lipid oxidation (determination of thiobarbituric acid and fluorescence indices) and hydrolysis (free fatty acids formation) in hake samples packaged in the alga-gelatine condition. A preservative effect resulting from the PH presence in the gelatine-based biofilm is concluded, this showing a quality and safety enhancement and a potential commercial value increase. Further research focused on the optimisation of the current biofilm preparation ought to be addressed.

Keywords: gelatine film; *Fucus spiralis*; protein hydrolysate; refrigerated fish; lipid damage; microbial activity

INTRODUCTION

Marine foods deteriorate rapidly *post-mortem* as a consequence of a variety of biochemical and microbial breakdown mechanisms [1]. According to the increasing demand for high-quality fresh products, different strategies have been tested to delay fish damage as long as possible [2]. One strategy has been the use of packaging films containing preservative compounds (i.e., antimicrobials and antioxidants) so that a marine product with an increased shelf-life time is attained [3, 4]. Among preservative compounds, adverse health problems resulting of persistent consumption of synthetic antioxidants have recommended the use of natural antioxidants as an alternative to synthetic ones [5]. Therefore, the identification and isolation of novel natural antioxidants from aquatic and terrestrial sources is receiving an increasing interest.

Marine macroalgae have been reported to include a great variety of chemical constituents with potential antioxidant [6] and antimicrobial [7] activities susceptible to be applied during seafood processing. Thus, a wide number of biopreservative molecules such as polyphenols, phlorotannins, terpenes, chlorophylls, carotenoids, etc., have been isolated from such kind of algae. Among them, *Fucus spiralis*, a brown macroalga living in the littoral shore of the Atlantic coasts of Europe and North America, has shown promising preserving properties in recent studies [8, 9].

This work was focused on the development of novel active packaging methods based on natural compounds. Accordingly, its main objective was to investigate the potential preservative effects that the inclusion of a protein hydrolysate (PH) obtained from *F. spiralis* in a gelatine-based film may have on the preservation of hake (*Merluccius merluccius*) slices kept under refrigerated (4 ºC) conditions. For it, microbiological and chemical analyses related to quality loss were monitored throughout a 6-day storage.

MATERIALS AND METHODS

Film systems preparation

Lyophilised alga (*F. spiralis*) was provided by Porto-Muiños (Cerceda, A Coruña, Spain). A PH from this lyophilised alga was prepared following the procedure described by Benelhadj *et al*. [10] and later on incorporated into a cross-linked bovine gelatine film. For it, films were prepared by casting from their film-forming solutions (FFS). Thus, 50 g of alga PH:gelatine (1:24) powder were dissolved in 500 mL of 0.01 M NaOH and stirred for 20 min at 40 ºC. Oxidised sodium alginate (2.5 g; 5 % wt) and glycerol (7.5 g; 30 % wt) were then incorporated into the FFS as crosslinking agent and plasticiser, respectively. The suspension was stirred at 40 ºC for 120 min.

* To whom all correspondence should be sent:
E-mail: saubourg@iim.csic.es

Then, the FFS were cast onto teflon-coated trays and dried at 40 ºC in a convection oven for 48 h. The films were conditioned during 48 h in a chamber at 4±1 ºC prior to use. The resulting biofilm was referred to as FS-GE packaging condition.

A control gelatine biofilm was prepared in the same way as the FS-GE batch, but without *F. spiralis* PH (GE-CT packaging condition; gelatine control).

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

Fish material, processing and sampling

Fresh hake (*Merluccius merluccius*) (9 specimens; 3.7-4.2 kg each) were caught near the Galician Atlantic coast (North-western Spain) and transported to the laboratory. Throughout this process (10 h), the fish were maintained in ice.

Upon arrival to the laboratory, specimens were cut in order to obtain 63 slices of 115-125 g each. Nine of the slices were distributed into 3 batches (three slices per group) that were analysed separately as initial material (n = 3). Meantime, 18 slices were placed in open air in a refrigerated room (4 ºC) and were considered as the Control batch (CONT condition). The remaining 36 slices were divided into two groups (18 slices per group) and were sealed-packaged individually in the two above mentioned packaging conditions (GE-CT and FS-GE), respectively. Packaged samples were placed in the above-mentioned refrigerated room (4 ºC). Fish samples from all batches were stored under such condition for a 6-day period, being sampled and analysed on days 1, 4 and 6. At each sampling time, 6 slices were taken from each batch for analysis and divided into three groups (two slices in each group) that were studied independently (n = 3).

Analysis of microbial development

Portions of 10 g of fish muscle were dissected aseptically from refrigerated fish specimens, mixed with 90 mL of 0.1 % peptone water (Merck, Darmstadt, Germany) and homogenised in sterilised Stomacher bags (AES, Combourg, France) as previously described [11, 12]. Serial dilutions from the microbial extracts were prepared in 0.1 % peptone water in all cases.

Total aerobes were determined on plate count agar (PCA, Oxoid Ltd., London, UK) after incubation at 30 ºC for 48 h. Anaerobes were investigated in the same manner, except that an anaerobic atmosphere kit was placed, together with the plates, inside the anaerobiosis jar. Psychrotrophs were determined in PCA, after incubation at 7-8 ºC for 7 days. *Enterobacteriaceae* were investigated in Violet Red Bile Agar (VRBA) (Merck, Darmstadt, Germany) after incubation at 37±0.5 ºC for 24 h. Microorganisms exhibiting proteolytic or lipolytic phenotypes were investigated in casein-agar or tributyrine-agar, respectively, after incubation at 30 ºC for 48 h, as previously reported [13].

In all cases, bacterial counts were transformed into log $CFU \cdot g^{-1}$ muscle units before undergoing statistical analysis. All analyses were conducted in triplicate.

Analysis of lipid damage development

Lipids were extracted from the hake white muscle by the Bligh and Dyer [14] method, which employs a single-phase solubilisation of the lipids using a chloroform-methanol (1:1) mixture. The results were calculated as g $lipid \cdot kg^{-1}$ muscle.

Free fatty acid (FFA) content was determined using the lipid extract of the fish muscle by the Lowry and Tinsley [15] method, which is based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment (Beckman Coulter DU 640 spectrophotometer). The results were expressed as g $FFA \cdot kg^{-1}$ muscle.

Thiobarbituric acid index (TBA-i) was determined according to Vyncke [16]. 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 calculated as mg $malondialdehyde \cdot kg^{-1}$ muscle.

Fluorescent compounds formation (fluorimeter LS 45; Perkin Elmer España; Tres Cantos, Madrid, Spain) was measured in the aqueous fraction obtained from the lipid extraction [14]. As described previously [17], fluorescence was measured at excitation/emission of 393/463 and 327/415 nm. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission wavelength pair and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 $\mu g \cdot mL^{-1}$ in 0.05 M H_2SO_4) at the corresponding wavelength pair. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$.

Statistical analysis

Data obtained from all microbiological and chemical analyses were subjected to the ANOVA method to explore differences resulting from the effect of the packaging system. The comparison of means was performed using the least-squares difference (LSD) method. In all cases, analyses were carried out using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences among packaging batches were considered significant for a confidence interval at the 95 % level ($p < 0.05$) in all cases.

RESULTS AND DISCUSSION

Microbial development

Analysis of aerobe counts showed an increase with storage time in most cases (Table 1). However, in all batches under study, values obtained can be considered as acceptable from a safety point of view as being below 6 log units even after 6 days of storage. Comparison among batches showed lower average values in fish corresponding to the alga-treated batch when compared with their counterparts from both control conditions; interestingly, significant differences ($p < 0.05$) could be observed at day 4 when compared with fish from CONT condition.

Table 1. Development of aerobes, psychrotrophs and *Enterobacteriaceae* counts (log CFU·g^{-1} muscle)* in refrigerated hake stored under different packaging conditions**

Microbial group	Packaging condition	Refrigeration time (days)			
		Initial	1	4	6
Aerobes mesophiles	CONT	2.50 (0.46)	3.09 a (0.76)	3.89 b (0.27)	4.13 a (0.68)
	GE-CT		2.43 a (0.23)	3.81 ab (0.57)	3.53 a (0.68)
	FS-GE		2.20 a (0.17)	3.10 a (0.17)	3.50 a (0.17)
Psychrotrophs	CONT	2.36 (0.32)	2.70 a (0.20)	3.86 b (0.06)	4.50 ab (1.06)
	GE-CT		2.46 a (0.28)	3.22 ab (0.59)	3.95 b (0.10)
	FS-GE		2.40 a (0.17)	2.36 a (0.32)	3.30 a (0.30)
Enterobacteriaceae	CONT	BDL***	1.74 b (0.52)	1.07 a (0.12)	1.34 a (0.24)
	GE-CT		BDL a	1.39 a (0.35)	1.23 a (0.24)
	FS-GE		BDL a	1.07 a (0.12)	1.17 a (0.15)

* Average values of three replicates (n=3); standard deviations are indicated in brackets. For each refrigeration time, values accompanied by different letters (a, b) indicate significant differences (p<0.05); ** Packaging conditions: CONT (not packaged; control), GE-CT (biofilm including gelatine; gelatine control) and FS-GE (biofilm including gelatine and alga protein hydrolysate) in agreement to the Material and Methods section;***BDL: microbial numbers were below the limit of detection of the technique (1 log CFU·g^{-1} muscle).

Psychrotrophs counts revealed a progressive increase in fish corresponding to CONT and GE-CT batches (Table 1); however, fish belonging to the batch corresponding to the alga-treated fish only provided a marked increase at the end of the experiment. Comparison among batches showed higher average values for fish belonging to both control batches (CONT and GE-CT); a significant inhibitory effect ($p < 0.05$) of the alga PH presence in the gelatine film was obtained at days 4 and 6.

The assessment of *Enterobacteriaceae* counts did not provide a general trend along storage time (Table 1). No marked evolution and development of this microbial group was observed at any of the packaging conditions. However, comparison among batches showed lower average values when alga hydrolysate was present in the packaging system.

Development of proteolytic and lipolytic microorganisms showed a similar pattern throughout the storage time (Table 2); in most cases, a progressive formation could be observed in all batches. An inhibitory effect of the alga PH presence in the gelatine film was implied since lower average values were obtained in all cases in

fish corresponding to the FS-GE batch; interestingly, differences were found significant ($p < 0.05$) for lipolytic counts at day 1.

Anaerobes counts assessment showed a progressive formation with time in all kinds of samples (Table 2). Average values corresponding to alga-treated fish were found lower throughout the whole study when compared with their counterparts corresponding to both controls; differences were found significant ($p < 0.05$) at day 1.

A partial microbial activity inhibition can be implied as a result of the presence in the packaging system of the PH of the current alga. An inhibitory effect on microbial activity development of *Fucus* spp. and other kinds of macroalgae has been reported to be caused by the presence of terpenes and polyphenols, among other compounds [7]. In a previous study, lyophilised *F. spiralis* was included in a polylactic-based film, this leading to a reduced microbial development in megrim (*Lepidorhombus whiffiagonis*) fillets kept under refrigerated (4 ºC) condition for 11 days [9].

The inclusion of other macroalgae extracts in biofilms has also provided antimicrobial activity. This is the case of the red *Gelidium corneum*, whose presence in an edible film also including persimmon peel and grape fruit seed extracts, improved the physical properties and provided antimicrobial activity [18]. Furthermore, the presence of polyhydroxybutyrate and phenolic compounds in microalga *Spirulina platensis* protein isolates led to a marked inhibitory effect when included in an edible packaging system [19, 20].

Lipid oxidation development

A marked and progressive formation of secondary lipid oxidation compounds was observed in all kinds of samples throughout the storage time (Table 3). Surprisingly, a higher ($p < 0.05$) level was determined in fish corresponding to the FS-GE batch at day 1. However, lower average values were detected for the 4-6-day period for alga-treated fish when compared with samples belonging to both control batches.

A great formation of fluorescent compounds was observed at day 1 in all kinds of samples (Table 3); after that time, a slight decrease in their content was implied till the end of the experiment in most cases. Comparison among samples provided lower average values in all cases for fish samples corresponding to the batch including the alga PH in the gelatine-packaging system. Interestingly, differences with both controls were found significant ($p < 0.05$) at day 6; at that time, a lower ($p < 0.05$) value was obtained for the gelatine control when compared with the CONT batch.

In agreement to the results obtained, a partial antioxidant behaviour can be accorded to the PH obtained from alga *F. spiralis* when included in a gelatine-film system. An antioxidant effect of *F. spiralis* extract has already been proved in different *in-vitro* tests [21], showing a marked content on polyphenols [8] and α-tocopherol [22]. Furthermore, the presence of lyophilised *F. spiralis* in a polylactic acid packaging film also showed a marked antioxidant effect during the refrigerated storage (11 days at 4 ºC) of megrim (*Lepidorhombus whiffiagonis*) fillets [9].

Previous research also accounts for an antioxidant effect as a result of the inclusion in biofilms of extracts obtained from other macroalgae. Thus, alginate-based films prepared from a red macroalga (*Sargassum fulvellum*) provided antioxidant properties (ABTS and DPPH assays) to a biofilm also including black chokeberry [23]. Moreover, Carissimi *et al*. [24] reported the antioxidant properties of a starch-based film including an ethanolic extract of microalgae *Heterochlorella luteoviridis* and *Dunaliella tertiolecta*; this effect, determined as a thiobarbituric acid index decrease, was observed in salmon fillets stored at 6±2 ºC for 6 days.

Lipid hydrolysis formation

FFA content showed a marked and progressive increase throughout the whole study for all kinds of samples (Table 3). Comparison among batches showed lower values ($p < 0.05$) in fish samples that were packaged in gelatine films including the alga PH presence during the 4-6-day period.

Both endogenous enzyme activity and microbial activity have been signalled as responsible for FFA formation during the refrigerated storage of fish [2, 25]. Before the end of the microbial lag phase, endogenous enzyme activity should be predominant; after that time, microbial activity should gain importance and be mostly responsible for the development of lipid hydrolysis. Current results obtained on FFA content evolution, i.e., lower formation as a result of the alga PH presence, can be explained on the basis of the above-mentioned inhibitory effect on lipolytic counts development (Table 2) observed throughout the whole study.

Table 2. Development of proteolytics, lipolytics and anaerobes counts (log CFU·g^{-1} muscle)* in refrigerated hake stored under different packaging conditions**

Microbial group	Packaging condition	Refrigeration time (days)			
		Initial	1	4	6
Proteolytics	CONT	2.26 (0.24)	2.76 b (0.15)	3.43 a (0.51)	3.50 a (0.70)
	GE-CT		2.43 ab (0.51)	3.32 a (0.55)	3.37 a (0.47)
	FS-GE		2.10 a (0.17)	3.20 a (0.17)	2.69 a (0.36)
Lipolytics	CONT	BDL***	2.91 c (0.23)	2.72 a (0.39)	3.47 a (0.77)
	GE-CT		2.39 b (0.18)	2.36 a (0.32)	2.89 a (0.11)
	FS-GE		BDL a	2.36 a (0.39)	2.56 a (0.24)
Anaerobes	CONT	BDL***	2.39 b (0.35)	2.60 a (0.30)	3.46 a (0.15)
	GE-CT		2.20 b (0.17)	2.36 a (0.32)	3.20 a (0.17)
	FS-GE		BDL a	2.16 a (0.28)	2.85 a (0.55)

* Average values of three replicates (n = 3); standard deviations are indicated in brackets. For each refrigeration time, values accompanied by different letters (a, b) indicate significant differences ($p < 0.05$). ** Packaging conditions as expressed in Table 1. ***BDL: microbial numbers were below the limit of detection of the technique (2 log CFU·g^{-1} muscle).

Table 3. Assessment of lipid damage* in refrigerated hake stored under different packaging conditions**

Chemical quality index	Packaging condition	Refrigeration time (days)			
		Initial	1	4	6
Thiobarbituric acid index **(mg malondi-aldehyde· kg^{-1} muscle)	CONT	0.09 (0.10)	0.12 a (0.01)	0.79 b (0.10)	1.48 a (0.98)
	GE-CT		0.22 b (0.01)	0.60 ab (0.07)	0.96 a (0.29)
	FS-GE		0.38 c (0.05)	0.43 a (0.08)	0.61 a (0.34)
Fluorescence ratio	CONT	1.25 (0.87)	5.76 b (0.57)	5.08 a (0.37)	5.00 c (0.24)
	GE-CT		4.92 ab (0.59)	5.18 a (0.48)	4.61 b (0.18)
	FS-GE		4.69 a (0.36)	4.37 a (0.50)	3.91 a (0.14)
Free fatty acids content (g·kg^{-1} muscle)	CONT	51.99 (4.95)	132.73 b (15.48)	338.00 b (58.95)	390.70 b (16.27)
	GE-CT		106.26 ab (10.47)	312.27 b (13.29)	381.96 b (14.43)
	FS-GE		77.30 a (21.14)	264.36 a (23.28)	339.53 a (14.21)

* Average values of three replicates (n = 3); standard deviations are indicated in brackets. For each refrigeration time, values accompanied by different letters (a, b, c) indicate significant differences ($p < 0.05$). ** Packaging conditions as expressed in Table 1.

Previous studies on the effects of algae extracts or any other alga-derivate can be considered as scarce. Thus, Taghavi Takyar *et al*. [26] reported the inhibitory effect of ethanol extracts of *S.*

platensis on lipid hydrolysis (FFA formation); such extract was added to rainbow trout (*Oncorhynchus mykiss*) fillets that were packaged in polyethylene bags and kept at 4 ºC up to 16 days. Related to gelatine-film systems, an inhibitory effect on lipid hydrolysis during fish products storage has also been reported. Thus, a chitosan-gelatine coating slowed down FFA formation in rainbow trout (*O. mykiss*) fillets [27] and Belanger's croaker (*Johnius balangerii*) fillets [28] during refrigerated storage (4 ± 1 ºC). Interestingly, a marked direct effect of FFA formation has been reported to be exerted on lipid oxidation, this effect being explained on the basis of a lower oxidative stability in FFA than in their corresponding methyl esters and triacylglycerides [29].

CONCLUSIONS

A novel gelatine-based film including a *F. spiralis* PH was tested as packaging method for the preservation under refrigerated conditions of hake slices. In it, a progressive quality loss could be observed in hake muscle by increasing the storage time. However, comparison with control fish showed a partial inhibitory effect on microbial activity development (counts assessment of aerobes, psychrotrophs, *Enterobacteriaceae*, proteolytics, lipolytics and anaerobes) and lipid oxidation (determination of thiobarbituric acid and fluorescence indexes) and hydrolysis (free fatty acids formation) evolution. A preservative effect resulting from the PH presence in the gelatine-based biofilm is concluded, this showing a quality and safety enhancement and a potential commercial value increase. Further research focused on the optimisation of the current biofilm preparation ought to be addressed.

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