Food Control

Antimicrobial and antioxidant effect of macroalga Fucus spiralis addition on gelatin film during refrigerated storage of mackerel

--Manuscript Draft--

Manuscript Number:	FOODCONT-D-21-00505R1
Article Type:	Research Paper
Keywords:	Fucus spiralis; gelatin packaging; Scomber scombrus; Refrigeration; microbial development; lipid damage
Corresponding Author:	Santiago P. Aubourg, Ph.D. Consejo Superior de Investigaciones Cientificas Vigo, SPAIN
First Author:	Marcos Trigo
Order of Authors:	Marcos Trigo
	Pedro Nozal
	José M. Miranda
	Santiago P. Aubourg, Ph.D.
	Jorge Barros-Velázquez
Abstract:	Lyophilized alga Fucus spiralis powder was incorporated into a gelatin-based film and employed as a packaging system for mackerel (Scomber scombrus) muscle portions throughout a 9-day refrigerated storage period at 4 ºC. In global terms, a progressive loss of quality could be observed in fish muscle with increasing storage time. Comparisons between batches allowed us to conclude an inhibitory effect of F. spiralis-containing films on microbial activity (assessment of aerobes, psychrotrophs, and proteolytic bacteria) and on lipid hydrolysis (as determined by free fatty acid formation) in mackerel muscle. The presence of the lyophilized macroalga in the packaging film also led to a higher retention of primary (peroxides) and secondary (thiobarbituric acid reactive substances) lipid oxidation compounds, while the formation of fluorescent compounds (interaction compounds between lipid oxidation compounds and nucleophilic molecules present in the fish muscle) decreased. Both antimicrobial and antioxidant effects were more intense when the concentration of alga in the packaging film was increased. The preservative effect resulting from the presence of F. spiralis in gelatin-based films demonstrates the potential employment of such bioactive films to improve the retention of fish quality and enhance its commercial value.

Food Control journal
Editorial Board

Vigo, June 3rd 2021

Dear Ms Ichiko Charis Howells:

Please find enclosed the manuscript now entitled **"Antimicrobial and antioxidant effect of lyophilized *Fucus spiralis* addition on gelatin film during refrigerated storage of mackerel"**. It has been performed according to the Reviewers' recommendations and criticisms. Changes made are marked on the text. We would like to submit it again for publication in the *Food Control* journal.

Yours sincerely,

Prof. Santiago P. Aubourg
Marine Research Institute
Spanish National Research Council (CSIC)
Vigo, Spain

CHANGES MADE AND COMMENTS

Reviewer #2:
In this paper, the authors made an attempt to evaluate edible biofilm prepared by incorporating the the lyophilized alga Fucus spiralis into a gelatin and evaluated as a packaging system for mackerel (Scomber scombrus) fillets under refrigerated (4 ºC) storage period for 9 days. The manuscript is well written and contains some interesting information's also. However, the manuscript contains some technical drawbacks that are really in need for its improvement. The paper quality can be improved by modifying the manuscript with following suggestions

1) The treatments groups are very widely in concentrations (1:5 and 1:20 alga to gelatin ratio) why the authors are not tried intermediate concentrations
Answer: As stated in sub-section 2.1., the highest alga concentration (i.e., 1:5 alga to gelatine ratio) was chosen to be employed on the basis of preliminary trials. This ratio was the highest that did not show any negative effect on sensory properties of fish muscle portions. Then, in order to cover a wide range of ratios, a relatively lower ratio (i.e. 1:20) was considered.

2) There is no information about bioactive properties of the biofilm.
Answer: The bioactive properties of the biofilm constituents are now included in the revised manuscript, following the Reviewer's advice. Thus, preservative properties of gelatin films have been described in the Introduction section of the revised manuscript. On the other side, preservative properties of F. spiralis constituents (hydrophilic and lipophilic) and their application to different kinds of seafood, this including lyophilized F. spiralis in biodegradable films (i.e. polylactic acid), are now included in the revised manuscript. In the present study, gelatin packaging was considered as control. Consequently, the Results and Discussion section shows comparison with previous research including hydrophilic and/or lipophilic constituents included in F. spiralis and other related macroalgae.

3) Which compounds are responsible for its bioactive properties?
Answer: The current study focused on the direct application of this macroalga in order to evaluate the possible preservative effects. No further analytical study was accomplished on the F. spiralis composition. However, and according to previous results, both bioactive hydrophilic and lipophilic compounds are known to be present in the current macroalga as well as in related macroalgae. Accordingly, information and discussion concerning previous studies on bioactive compounds (antimicrobial and antioxidant properties) are now included in the Results and Discussion section, as suggested by the Reviewer.

4) What about the colour of film? Does it imparts colour to the fish fillet?
Answer: As stated in sub-section 2.1, the most concentrated alga:gelatin ratio provided colourless films which did not modify the sensory properties of the muscle portions (colour, odour, or taste)

5) Sensory parameters also need to be discussed
Answer: In the current study, we focused on microbial and chemical indices related to microbial breakdown and lipid damage. Sensory acceptance was not explicitly evaluated. However, a strong direct relationship between microbial counts and sensory acceptance of refrigerated fish is widely accepted. This is mentioned now in the revised manuscript. Furthermore, lipid oxidation development has also shown a direct effect on sensory acceptance. This is also included now in the Results and Discussion section of the revised manuscript.
Some new references have been included now concerning the relationship between such quality parameters.

Reviewer #3: General comments:
This article has interesting results that can contribute for fish gelatin-based films. The research topic is interesting and the results could have interest for the food industry. However, the experimental has some week points: only one parameter to evaluate the legal limits for fish quality was used (Total viable counts). Given the potential benefice of this type of films, other parameters should be analysed to have conclusions that are more realistic and of interest to the sector.
Answer: Thanks for your comment. Results on Enterobacteriaceae counts have been included now in the revised manuscript. In the previous version of the manuscript this determination was not included. It should be highlighted that, values remained in all cases below 1 log CFU/g. This microbial parameter has a widely accepted limit of 3 log CFU/g for fish. As stated in the revised manuscript now, all batches were acceptable throughout the whole study on the basis of this determination.
Concerning the lipid oxidation assessment, a complete lipid oxidation analysis (three levels) was undertaken on the basis that a fatty fish species was concerned. We agree with the Reviewer in that such determinations do not provide legal limit values established for all kinds of fish products; however, the assessment of such indices are widely accepted to provide a useful information of quality loss in refrigerated fish.

Additionally, sensory parameters should be considered.
Answer: In the current study, we focused on microbial and chemical indices related to microbial breakdown and lipid damage. Sensory acceptance was not explicitly evaluated. However, a strong direct relationship between microbial counts and sensory acceptance of refrigerated fish is widely accepted. This is mentioned now in the revised manuscript. Furthermore, lipid oxidation development has also shown a direct effect on sensory acceptance. This is also included now in the Results and Discussion section of the revised manuscript.
Some new references have been included now concerning the relationship between such quality parameters.

Revision comments:
- The abstract should be revised. In the Abstract, it is not clear if the authors use lyophilized alga Fucus spiralis to incorporate into a gelatin-based film or macroalga extract.

Answer: The first sentence in Abstract section was modified according to the Reviewer's comment. Later on, "macroalga extract" has been replaced with "lyophilized macroalga". The title of the manuscript was also modified to better express that lyophilized alga was employed.

- Materials and methods: Fish material, processing, and sampling

This section is vague and must be improved.
Answer: Description of this section has been improved. The term "fillets" has been eliminated and replaced with "muscle portions" or "fish portions".

Line 124: what type of gelatin was used? gelatin obtained from fish?
Answer: Information about gelatin (i.e. from fish origin) has been included now in the revised manuscript, as suggested by the Reviewer.

Line 152: please explain how the samples were sealed-packaged individually under the three above-mentioned packaging systems.
Answer: Information about packaging has been improved in the revised manuscript. The size of each pack is also included now in the revised manuscript.

HIGHLIGHTS

- Lyophilized *Fucus spiralis* was incorporated into a gelatin packaging film
- *F. spiralis* in the packaging film inhibited free fatty acid formation in mackerel
- *F. spiralis* inhibited aerobes, psychrotrophs, and proteolytic bacteria in fish muscle
- *F. spiralis* in the packaging film inhibited fluorescent compound formation
- Superior retention of primary and secondary lipid oxidation compounds was observed

Antimicrobial and antioxidant effect of lyophilized *Fucus spiralis* addition on gelatin film during refrigerated storage of mackerel

Marcos Trigo[1], Pedro Nozal[1], José M. Miranda[2], Santiago P. Aubourg[1,*], and Jorge Barros-Velázquez[2]

[1] Department of Food Science and Technology, Marine Research Institute (CSIC), c/ E. Cabello, 6 36208-Vigo, Spain

[2] Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences, University of Santiago de Compostela, Avenida Carvallo Calero, s/n, 27002-Lugo, Spain

[*] Correspondent: saubourg@iim.csic.es; +34986292762 (fax), +34986231930 (phone)

ABSTRACT

Lyophilized alga *Fucus spiralis* powder was incorporated into a gelatin-based film and employed as a packaging system for mackerel (*Scomber scombrus*) muscle portions throughout a 9-day refrigerated storage period at 4 ºC. In global terms, a progressive loss of quality could be observed in fish muscle with increasing storage time. Comparisons between batches allowed us to conclude an inhibitory effect of *F. spiralis*-containing films on microbial activity (assessment of aerobes, psychrotrophs, and proteolytic bacteria) and on lipid hydrolysis (as determined by free fatty acid formation) in mackerel muscle. The presence of the lyophilized macroalga in the packaging film also led to a higher retention of primary (peroxides) and secondary (thiobarbituric acid reactive substances) lipid oxidation compounds, while the formation of fluorescent compounds (interaction compounds between lipid oxidation compounds and nucleophilic molecules present in the fish muscle) decreased. Both antimicrobial and antioxidant effects were more intense when the concentration of alga in the packaging film was increased. The preservative effect resulting from the presence of *F. spiralis* in gelatin-based films demonstrates the potential employment of such bioactive films to improve the retention of fish quality and enhance its commercial value.

Keywords: *Fucus spiralis*; gelatin packaging; *Scomber scombrus*; refrigeration; microbial development; lipid damage

Running title: Alga-gelatin packaging and mackerel quality

1. INTRODUCTION

The consumption of marine species provides many benefits to human health, as they are important sources of polyunsaturated fatty acids (PUFA), high-quality proteins, minerals, and lipophilic vitamins (Tilami & Sampels, 2018). However, their high water and non-protein nitrogen contents, soft muscular and skin structure, low content of connective tissue, and poikilothermic nature make marine species perishable commodities (Özoğul, 2010). Lowering the temperature with ice or mechanical refrigeration is the most common way of retarding microbial and biochemical spoilage of fish. Although widespread, neither technique can guarantee the retention of fish quality, especially when relatively long storage times are applied or when the cold chain is not strictly maintained. Consequently, advances in refrigeration processes have been developed to circumvent these scenarios (Campos, Gliemmo, Aubourg, & Barros-Velázquez, 2012).

One such preservation technology is packaging. Packaging maintains food freshness and preserves foods during distribution and storage from adverse situations, including water vapor, microorganisms, gases, odors, dust, and mechanical shock and vibrations (Mihindukulasuriya & Lin, 2014; Dehghani, Hosseini, & Regenstein, 2018). Interestingly, currently existing packaging technologies work together with a number of physical, chemical, and biological processes and agents to limit both microbial activity and biochemical breakdown in foods (Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013; Kuley, Özoğul, & Polat, 2020).

Biodegradable and edible materials derived from plants and animals, including peptides, polysaccharides, and lipids, are profitable alternatives to synthetic packaging films (Umaraw et al., 2020). Among the polysaccharides, gelatin obtained from diverse animal sources (porcine, bovine, and fish) has been applied effectively in active packaging strategies for its film-forming ability (Etxabide, Uranga, Guerrero, & De la Caba, 2017). Thus, gelatin-based coating and packaging films reduce oxygen, oil, and moisture transport (Pavli et al., 2018) and can be used to reduce oxidation events, preserve flavor, and improve the color stability, taste, and aroma of foods (Cuter, 2006). Furthermore, the combination of gelatin packaging films with different kinds of natural preservative compounds from different sources, such as oregano and rosemary extracts (Gómez-Estaca, Montero, Giménez, & Gómez-Guillén, 2007), oregano oil (Min & Oh, 2009), tea polyphenols (Feng, Ng, Mikš-Krajnik, & Yang, 2017), or a microalga protein concentrate (Stejskal, Miranda, Martucci, Ruseckaite, Barros-Velázquez, & Aubourg, 2020a), have successfully improved the retention of seafood quality by inhibiting both microbial activity and lipid oxidation events.

Marine macroalgae have been reported to contain a wide variety of chemical constituents with potential antimicrobial and antioxidant activities (Sandsdalen, Haug, Stensvag, & Styrvold, 2003; Gupta & Abu-Ghannam, 2011), potentially applicable to seafood processing. Thus, a wide variety of bioactive compounds, such as polyphenols, pholorotannins, terpenes, chlorophylls, and carotenoids, have been isolated from different algae species. Among them, *Fucus spiralis*, a brown macroalga living on the littoral shore of the Atlantic coasts of Europe and North America, has shown promising preservation potential (Farvin & Jacobsen, 2013; Tierney, Smyth, Hayes, Soler-Vila, Croft, & Brunton, 2013a; Andrade et al., 2013). Thus, its presence in a polylactic acid-based film employed for megrim (*Lepidorhombus whiffiagonis*) refrigerated storage (García-Soto et al., 2015) and in the icing medium employed during hake (*Merluccius merluccius*) (Barros-Velázquez, Miranda, Ezquerra-Brauer, & Aubourg, 2016) storage leads to substantial quality enhancement of fish specimens. In the present study, lyophilized *F. spiralis* was incorporated into a gelatin-based packaging film and employed for the preservation of mackerel (*Scomber scombrus*) muscle portions during refrigerated (4 ºC) storage. The potential preservative effect derived from the presence of alga in the packaging system was monitored by microbiological and chemical analyses throughout a 9-day storage period, as compared with a control batch.

2. MATERIALS AND METHODS

2.1 Film system preparation

Lyophilized alga (*F. spiralis*) was provided by Porto-Muiños (Cerceda, A Coruña, Spain). Teleostean gelatin (Sigma, Life Sciences, Steinheim, Germany) films were produced by casting from their fill-forming solutions (FFS) according to Stejskal et al. (2020a). Two kinds of biofilms were prepared to test two different concentrations of lyophilized alga. For the less concentrated preparation, 50 g of a combined alga:gelatin (1:20) powder were dissolved in 500 mL of 0.01 M NaOH and stirred for 20 min at 40 ºC. Oxidized sodium alginate (2.5 g; 5% wt.) and glycerol (7.5 g; 30% wt.) were then incorporated into the FFS as crosslinking agent and plasticizer, respectively. The resulting suspension was stirred at 40 ºC for 120 min. 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 for 48 h in a chamber at 4±1 ºC prior to use. The resulting biofilm was referred as the F-1 packaging film and batch.

To prepare the most concentrated packaging film, a similar procedure was carried out but starting from 50 g of a combined alga:gelatin (1:5) powder. The resulting biofilm and subsequent batch were referred as the F-2. A control gelatin film without alga was prepared in the same way as the F-1 and F-2 packaging films and referred to as the CT film and batch.

The contents of lyophilized alga chosen in the current study were established on the basis of previous trials carried out in our laboratory (data not shown). Thus, a 1:5 alga:gelatin ratio was the highest alga concentration that did not modify the sensory and external features of fish muscle portions (i.e., odor, color). Consequently, this concentration was considered, together with a less concentrated one (1:20 ratio).

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

2.2 Fish material, processing, and sampling

Fresh Atlantic mackerel (50 specimens; 225–270 g each) were caught near the Galician Atlantic coast (northwestern Spain) and transported to the laboratory. Throughout this process (10 h), the fish specimens were maintained in ice.

Upon arrival in the laboratory, specimens were beheaded, eviscerated, skinned, filleted and cut in order to obtain 90 fish muscle portions of 65–75 g each. Nine such portions were distributed into three groups (3 portions per group) that were analyzed separately as initial material (*n* = 3). The remaining 81 fish portions were divided into 3 groups (27 portions per group), and were sealed-packaged under the three above-mentioned packaging systems (CT, F-1, and F-2, respectively). Each fish portion was packaged individually (10 x 15 cm). Packaged muscle portions were stored in a refrigerated room (4 ºC) for a 9-day period, sampling and analyses being performed on days 2, 6, and 9. At each sampling time, 9 fish portions were taken for analysis from each batch and divided into 3 groups (3 portions for each group), which were studied independently (*n* = 3).

2.3 Microbiological analyses

Muscle samples of 10 g were taken aseptically from chilled fish portions and homogenized with 90 mL of 0.1% peptone water (Merck, Darmstadt, Germany) in sterile stomacher bags (AES, Combourg, France) as previously described (Sanjuás-Rey, García-Soto, Fuertes-Gamundi, Aubourg, & Barros-Velázquez, 2012; García-Soto, Aubourg, Calo-Mata, & Barros-Velázquez, 2013). Aerobes were investigated on plate count agar (PCA, Oxoid Ltd., London, UK), incubation being carried out for 48 h at 30 ºC. Anaerobic bacteria were determined under the same conditions, with the exception that an anaerobic atmosphere kit was placed, together with the plates, inside the anaerobiosis jar. *Enterobacteriaceae* were investigated in Violet Red Bile Agar (VRBA) (Merck, Darmstadt, Germany) after incubation at 37 ± 0.5 °C for 24 h. Psychrotrophic bacteria were counted in PCA, after an incubation period of 7 days at 7–8 ºC. Microorganisms able to produce proteolytic or lipolytic extracellular enzymes were determined on casein-agar or tributyrine-agar, respectively, incubation being carried out for 48 h at 30 ºC, as previously reported (Rodríguez et al., 2005).

For all microbiological analyses, bacterial counts were converted into log CFU·g^{-1} before statistical analysis was performed. All analyses were conducted in triplicate.

2.4 Analysis of lipid damage development

Lipids were extracted from mackerel white muscle by the Bligh and Dyer (1959) method, which employs a single-phase solubilization of lipids using a chloroform-methanol (1:1) mixture. The results were calculated in g lipid·kg^{-1} muscle.

Free fatty acid (FFA) content was determined using lipids extracted from fish muscle by the Lowry and Tinsley (1976) method, which is based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment

(Beckman Coulter DU 640 spectrophotometer, Beckman Coulter Inc., Brea, CA, USA). The results were calculated as g FFA·kg^{-1} muscle.

The peroxide value (PV) was determined spectrophotometrically (520 nm) on the lipid extract by peroxide reduction with ferric thiocyanate, according to Chapman and McKay (1949). The results were calculated as meq active oxygen·kg^{-1} lipids.

The thiobarbituric acid index (TBA-i) was determined according to Vyncke (1970). This method is based on the reaction between a trichloroacetic acid extract of the fish muscle and thiobarbituric acid. The content of thiobarbituric acid reactive substances (TBARS) was measured spectrophotometrically at 532 nm and calculated from a standard curve using 1,1,3,3-tetraethoxy-propane (TEP). The results were calculated as mg malondialdehyde·kg^{-1} muscle.

Fluorescent compound formation (Fluorimeter LS 45; Perkin Elmer España; Tres Cantos, Madrid, Spain) was measured in the aqueous fraction obtained from the lipid extraction process. As described previously (Aubourg, 1999), fluorescence was measured at excitation/emission at 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 sulfate solution (1 µg·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 = $RF_{393/463\ nm}/RF_{327/415\ nm}$.

2.5 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 and the refrigeration time. Means were compared 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 were considered significant for a confidence interval at the 95% level ($p<0.05$) in all cases.

3. RESULTS AND DISCUSSION

3.1 Effect of the bioactive films on microbial growth in mackerel

Microbiological analyses were performed in all three fish batches along the 9 days of refrigerated storage. With respect to the development of aerobic bacteria, the F-2 batch, which included the highest alga concentration in the packaging film, allowed for better control of the growth of this microbial group (Figure 1). Thus, a 0.9 log CFU·g^{-1} significant ($p<0.05$) difference between the CT and F-2 batches was determined on day 9, thus indicating that the aerobe load in the latter batch was nearly 10 times lower as compared with the CT batch. With respect to psychrotrophs, a similar behavior was observed, but in this case both F-1 and F-2 batches exhibited significantly ($p<0.05$) lower numbers than the CT batch after 9 days of refrigerated storage (Figure 2). The best control of psychrotrophic growth in mackerel muscle was determined in the F-2 batch on day 9, in which the load of this microbial group was 0.43 log units below that determined in the CT batch. The current study also includes the comparative analysis of *Enterobacteriaceae* growth in all three batches. The presence of this bacterial group was very limited in all cases, with microbial counts being always below 1 log CFU·g^{-1}, a level far below the widely accepted limit of 3 log CFU·g^{-1}.

Microorganisms exhibiting a specific spoilage phenotype, namely proteolytic or lipolytic activity, were also investigated in all three batches. With respect to bacteria able to produce extracellular proteases, the effect of the presence of the lyophilized alga in the packaging film allowed for better control of this microbial group throughout refrigerated storage, as compared with the control batch (Table 1). Thus, average counts of proteolytic bacteria on day 9 were lower in both F-1 and F-2 batches as compared with the CT batch. Nonetheless, such differences only proved to be significant ($p<0.05$) when comparing the F-1 and CT batches (0.83 log units lower in the F-1 batch, Table 1). With respect to the development of bacteria able to produce extracellular lipases, the results indicated that the counts of this microbial group were in all cases below 3 log CFU·g^{-1} (except for the CT batch on day 9), thus indicating very limited development of lipolytic bacteria in mackerel muscle regardless of the packaging system considered (Table 1). However, on day 9, the average counts determined in the F-1 and F-2 batches were below those determined for the CT batch, although such differences were not found to be significant ($p>0.05$). Similar results were observed for the anaerobes, with numbers being in all cases below 3 log CFU·g^{-1}, thus indicating very limited growth of this microbial group in all three batches (Table 1). However, as in the case of lipolytic bacteria, the load of this microbial group was found to be lower in both the F-1 and the F-2 batches on day 9 as compared with the CT batch.

The results of microbiological analyses in the present study indicated partial microbial inhibition derived from the inclusion of the lyophilized *F. spiralis* in the packaging film (F-1 and F-2 batches). This inhibition was more intense in the case of the F-2 batch, which included a higher alga concentration in the packaging film. According to the direct relationship between microbial quality and sensory acceptance (Ólafsdóttir & Jónsdóttir, 2010; García-Soto et al., 2013), the microbial quality enhancement of fish muscle portions would imply increased sensory acceptance on the basis of the value increase of descriptors such as odor, taste, and color.

The inhibitory effect of *Fucus* spp. and other macroalgae species on microbial growth has been linked to the presence of different kinds of bioactive compounds. On one side, hydrophilic compounds such as sulfate polysaccharides, proteins, peptides, glycosides, low-molecular organic acids and salts have been reported to be present in seaweed and to exhibit potential preservative properties (Kuda & Ikemori, 2009; Pereira, Amado, Critchley, Van de Velde, & Ribeiro-Claro, 2009). Interestingly, water extracts of *F. spiralis* include preserving phenolic acids such as chlorogenic acid, vanilic and caffeic acid (Farvin & Jacobsen, 2013). On the other side, lipophilic compounds such as terpenes and polyphenols (Sandsdalen et al., 2003), halogenated alkane and alkenes, alcohols, aldehydes, hydroquinones, and ketones (Smit, 2004) and oligomeric phlorotannins (Serrano, Puupponen-Pimia, Dauer, Aura, & Saura-Calixto, 2009) have also been reported to be present in macroalgae and to exhibit preservative properties. This preservative 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 the inhibition of oxidative phosphorylation, and complexation of metal ions in the bacterial environment (Sandsdalen et al., 2003; Smit, 2004).

Previous studies accounted for an inhibitory effect of *F. spiralis* bioactive compounds on microbial activity in fish muscle during refrigerated storage. Thus, lyophilized *F. spiralis,* when included in a polylactic-based packaging film, inhibited the growth of aerobes, psychrotrophs, and *Enterobacteriaceae* in megrim (*Lepidorhombus whiffiagonis*) fillets stored at 4 ºC for 11 days (García-Soto et al., 2015). Furthermore, the presence of a *F. spiralis* extract in the icing medium employed for the chilled storage of hake (*Merluccius merluccius*) (Barros-Velázquez et al., 2016) and megrim (*Lepidorhombus whiffiagonis*) (Miranda, Trigo, Barros-Velázquez, & Aubourg, 2016) allowed for better control of aerobic, psychrotrophic, proteolytic, and lipolytic bacteria.

The inclusion of other algae or algae extracts in biofilms has also been reported to provide antimicrobial activity. This is the case of the red macroalga *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 (Jo, Song, Lee, & Song, 2014). Furthermore, the presence of polyhydroxybutyrate and phenolic compounds extracted from microalga *Spirulina platensis* led to a marked inhibitory effect of microbial activity when included in an edible packaging system (Goettems Kuntzler, Araujo de Almeida, Vieira Costa, & Greque de Morais, 2018). Remarkably, recent studies have reported microbial quality enhancement of refrigerated (4 ºC) hake (*M. merluccius*; Stejskal et al., 2020a) and mackerel (*S. scombrus*; Stejskal, Miranda, Martucci, Ruseckaite, Aubourg, & Barros-Velázquez, 2020b) when a protein concentrate from *S. platensis* was included in the gelatin-based packaging film.

3.2 Determination of lipid hydrolysis development

FFA formation was found to be negligible ($p>0.05$) in all batches after 2 days of refrigerated storage (Figure 3). However, remarkable lipid hydrolysis was detected in all mackerel batches after 6 days of storage, this being followed by a further increase ($p<0.05$) at advanced storage times. A batch comparison showed an inhibitory effect on FFA formation ($p<0.05$) in fish specimens corresponding to the F-2 batch for the 2–9-day storage period. Notably, no significant effect ($p>0.05$) was concluded for the less concentrated packaging film (F-1), although this batch showed lower average values for the 6–9-day period when compared with the control batch.

Lipid hydrolysis itself does not lead to nutritional losses. However, a direct relationship between FFA formation and sensory acceptance was detected (Özoğul, 2010; Campos et al., 2012). Thus, the accumulation of FFA may imply detrimental sensory properties and negatively affect the consumers' acceptability of seafood (Sikorski & Kolakowski, 2000). Among such negative modifications, texture changes and the development of off-odor and off-taste can be highlighted. Furthermore, a marked direct effect of FFA formation on lipid oxidation has been reported, this effect being explained on the basis of a lower oxidative stability of FFA as compared with their corresponding triacylglycerols and phospholipids as a result of lower steric hindrance to oxidative reactions (Aubourg, 2001). FFA formation in fish muscle during refrigerated storage has been explained as a result of endogenous and microbial enzyme activities (Campos et al., 2012). Before the end of the microbial lag phase, FFA formation should be mostly caused by endogenous enzyme activity (i.e., lipases and phospholipases); later on, microbial-based extracellular lipases should be the predominant mechanism of FFA generation. On the basis that strong development of FFA formation was observed in the present study at extended storage times (6–9-day period), microbial activity seemed to be the most relevant mechanism responsible for FFA formation. Consequently, the inhibition of FFA formation in the F-1 and F-2 batches can be explained on the basis of the above-mentioned inhibition of microbial growth. This inhibition could be justified by the presence in the lyophilized alga of preservative hydrophilic and lipophilic bioactive compounds, as stated in the previous sub-section.

Previous studies reporting the effects of algae extracts on FFA development have led to opposite results. Thus, enhanced lipid hydrolysis events were observed in chilled minced Atlantic mackerel (*S. scombrus*) previously treated with an aqueous extract of *Polysiphonia fucoides* (Babakhani, Farvin, & Jacobsen, 2016). Similar results were observed in chilled hake (*M. merluccius*) exposed to ice including a *F. spiralis* water extract (Barros-Velázquez et al., 2016). However, when an ethanol extract of *F. spiralis* alga was considered, no significant effect on FFA formation was observed in hake (*M.*

merluccius) (Barros-Velázquez et al., 2016) and megrim (*L. whiffiagonis*) (Miranda et al., 2016) during chilled storage. In agreement with the results obtained in the current study, Taghavi Takyar, Haghighat Khajavi, and Safari (2019) reported the inhibitory effect of ethanol extracts of *S. platensis* on lipid hydrolysis in rainbow trout (*Oncorhynchus mykiss*) fillets packaged in polyethylene bags and kept at 4 ºC for up to 16 days. Recently, an inhibitory effect of a crosslinked-gelatin film including a spirulina protein concentrate on FFA formation was observed in lean (hake, *M. merluccius*; Stejskal et al., 2020a) and fatty (mackerel, *S. scombrus*; Stejskal et al., 2020b) fish muscle.

3.3 Determination of lipid oxidation development

The stability of packaged mackerel muscle against rancidity was studied by means of primary (peroxide value), secondary (TBA-i), and tertiary (FR) oxidation compound formation.

No significant formation of peroxides ($p>0.05$) was detected after 2 days of storage in any of the batches under study (Table 2). In contrast, a general increase ($p<0.05$) in the peroxide content was observed after 6 days, this increase being especially relevant in the case of the F-2 batch. At the end of the storage period, significant peroxide formation ($p<0.05$) was detected in fish specimens corresponding to the CT and F-1 batches, while no differences ($p>0.05$) were observed in the F-2 batch. The presence of the lyophilized alga in the packaging film led to higher average peroxide values in specimens corresponding to the F-2 batch throughout the whole storage period; notably, differences were found to be significant ($p<0.05$) at day 6, as compared with their counterparts belonging to the CT and F-1 batches.

A progressive formation ($p<0.05$) of TBARS was detected in all batches throughout the storage period (Table 2). Increasing average TBARS values were observed as the presence of alga in the packaging film increased. Thus, fish portions corresponding to the control batch exhibited the lowest average values at all sampling times. Interestingly, fish muscle corresponding to the F-2 batch showed higher TBARS levels ($p<0.05$) than the counterpart CT batch throughout the storage period. Furthermore, fish samples corresponding to the F-1 batch exhibited higher TBARS concentrations ($p<0.05$) at days 2 and 9, as compared with their control counterparts.

A significant formation of fluorescent compounds ($p<0.05$) was observed at day 2 in all batches, this formation being of special relevance in the control batch (Table 2). Analysis of interaction compound formation revealed a further formation at day 6 in all batches; at this time, the highest increase ($p<0.05$) was observed in mackerel muscle corresponding to the F-1 batch. An additional increase of the average FR value was depicted in all batches at the end of the experiment. The highest average FR values were determined in mackerel samples corresponding to the control batch throughout the whole storage period; differences among batches were found to be significant ($p<0.05$) at day 2 with respect to the F-1 batch and in the 2–9-day period with respect to the F-2 batch.

Lipid oxidation has been recognized as a multi-step process where different molecular species are subsequently produced. Those produced in the earliest stages (i.e., peroxide compounds) are reported to be more unstable and susceptible to breakdown and to produce lower-molecular-weight compounds (i.e., carbonyl compounds). Finally, at the advanced stages of lipid oxidation, both peroxide and carbonyl compounds are susceptible to reactions with other molecules (mostly of the nucleophilic-type including groups like $-NH_2$, or -SH) present in the fish muscle, thus leading to the formation of fluorescent compounds (namely, tertiary lipid oxidation compounds) (Pokorný, 1981; Aubourg, 1999). In the present study, higher peroxides and TBARS contents were observed in fish samples corresponding to the F-1 and F-2 batches, these contents increasing as the presence of lyophilized alga in the packaging film increased. However, the fact that the content of advanced lipid oxidation compounds (detected by FR) was lower in fish corresponding to the F-2 batch allowed us to conclude an inhibitory effect of *F. spiralis* on the breakdown and reactivity of primary and secondary lipid oxidation compounds. As a result, a decreasing effect on the FR value was detected, this effect being more important in the F-2 batch, including the highest concentration of alga. Remarkably, fluorescent compound formation can be considered an indicator of protein damage, which negatively affects sensory descriptors such as texture and juiciness (Özogul, 2010; Rustad, 2010).

Algae in general have been described as important sources of antioxidant compounds (Gupta & Abu-Ghannam, 2011; Farvin & Jacobsen, 2013). According to their photosynthetic role, algae are exposed to a strong combination of light and oxygen. Consequently, their natural content on antioxidant substances has been reported to be responsible for the lack of structural damage in their organs (Smit, 2004). In the case of *F. spiralis*, an antioxidant capacity has already been proved in different *in vitro* tests (DPPH and FRAP analyses) (Cérantola, Breton, Gall, & Deslandes, 2006; Andrade et al., 2013; Peinado, Girón, Koutsidis, & Ames, 2014), due to its marked content of polyphenols (Tierney et al., 2013a) and α-tocopherol (Paiva et al., 2014). The identification of active antioxidant compounds supported the assumption that phlorotannins were also present in this macroalga (Tierney, Smyth, Rai, Soler-Vila, Croft, & Brunton, 2013b). Additionally, and as stated above, Farvin and Jacobsen (2013) reported the presence of antioxidant phenolic acids such as chlorogenic acid, vanilic and caffeic acid in the current alga (Farvin and Jacobsen, 2013).

In agreement with the present results, no effect on TBARS content and a higher PV was detected in chilled megrim (*L. whiffiagonis*) subjected to ice including a *F.*

spiralis extract (Miranda et al., 2016). Likewise, an inhibitory effect on fluorescent compound formation was also observed. Similarly, the inclusion of *F. spiralis* extracts in the icing medium did not exert a definite effect on peroxide and TBARS formation in chilled hake (*M. merluccius*) (Barros-Velázquez et al., 2016). Remarkably, the incorporation of the alga extract in the ice led to an inhibitory effect on fluorescent compound formation. Closely related to the current study, the presence of lyophilized *F. spiralis* in a polylactic acid packaging film also showed a marked inhibitory effect on fluorescent compound formation during refrigerated storage (11 days at 4 ºC) of megrim (*L. whiffiagonis*) fillets (García-Soto et al., 2015). Additionally, no effect on peroxide content was detected.

Previous research also accounted for an antioxidant effect derived from the inclusion of extracts from other algae in biofilms. 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 (Kim, Back, & Song, 2018). Moreover, Carissimi, Flôres, and Rech (2018) 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 TBA-i decrease, was observed in salmon fillets stored at 6±2 ºC for 6 days. Also related to a microalga, the presence of a protein concentrate from *S. platensis* in the packaging film led hake (*M. merluccius*) muscle to a higher PUFA retention rate during refrigerated storage (4 ºC) (Stejskal et al., 2020a). Finally, a lower formation of fluorescent compounds was detected in mackerel (*S. scombrus*) muscle packaged in a gelatin film including a protein concentrate from *S. platensis* stored at 4 ºC (Stejskal et al., 2020b).

4. CONCLUSIONS

A gelatin-based film including lyophilized alga *F. spiralis* was tested as a novel packaging method for the preservation of mackerel muscle under refrigeration. The inclusion of lyophilized alga in the packaging films exerted a remarkable inhibitory effect on microbial activity (aerobes, psychrotrophs, and proteolytic bacteria) and on lipid hydrolysis events (free fatty acid formation). Concerning lipid oxidation, the presence of the macroalga in the packaging film led to a higher retention of primary (peroxides) and secondary (TBARS) lipid oxidation compounds, while the formation of fluorescent compounds (interaction compounds between such lipid oxidation compounds and nucleophilic molecules present in the fish muscle) decreased. Consequently, a preservative effect on lipid oxidation development was concluded on the basis of the inhibition of oxidation compounds produced at advanced deteriorative stages in fish muscle (i.e., inhibition of tertiary lipid oxidation formation). The preservative effect was concluded to be more intense in the batch containing a higher alga concentration in the packaging film. In global terms, a preservative effect derived from the incorporation of *F. spiralis* in the gelatin-based film was concluded, as a consequence of both antimicrobial and antioxidant activities. According to the direct relationship of microbial and chemical quality indices with respect to sensory assessment, a favourable effect on sensory acceptance could be implied for the packaging system evaluated in this work.

This study opens the door to the potential employment of lyophilized *F. spiralis* in bioactive gelatin films for enhancing the quality and the potential commercial value of refrigerated fish species. Further research focused on optimization of the current biofilm preparation (i.e., the alga/gelatin ratio, fish species concerned, refrigeration time and temperature, etc.) should be considered to apply this active packaging strategy to a wide variety of fish species. Furthermore, sensory acceptance evaluation would be necessary in order to fulfil consumers' demand for high-quality fresh products subjected to minimal processing and not including chemical preservatives.

Acknowledgements

Lyophilized alga *F. spiralis* was provided by Porto-Muiños (Cerceda, A Coruña, Spain). This work was supported by the Xunta de Galicia (Galician Government, Spain) through the research project INNOVA-PEMES (IN848D), 026–IN848D–2020–1119362.

REFERENCES

Andrade, P., Barbosa, M., Pedro Matos, R., Lopes, G., Vinholes, J., Mouga, T., & Valentão, P. (2013). Valuable compounds in macroalgae extracts. *Food Chemistry, 138*, 1819–1828.

Aubourg, S. P. (1999). Review: Recent advances in assessment of marine lipid oxidation by using fluorescence. *Journal of the American Oil Chemists' Society, 76*, 409–419.

Aubourg, S. P. (2001). Fluorescence study of the prooxidant activity of free fatty acids on marine lipids. *Journal of the Science of Food and Agriculture, 81*, 385–390.

Babakhani, A., Farvin, K., & Jacobsen, C. (2016). Antioxidative effect of seaweed extracts in chilled storage of minced Atlantic mackerel (*Scomber scombrus*): effect on lipid and protein oxidation. *Food and Bioprocess Technology, 9*, 352–364.

Barros-Velázquez, J., Miranda, J. M., Ezquerra-Brauer, J. M., & Aubourg, S. P. (2016). Impact of icing systems with aqueous, ethanolic and ethanolic-aqueous extracts of alga *Fucus spiralis* on microbial and biochemical quality of chilled hake (*Merluccius merluccius*). *International Journal of Food Science and Technology, 51*, 2081–2089.

Bligh, E., & Dyer, W. (1959). A rapid method of total extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*, 911–917.

Campos, C., Gliemmo, M., Aubourg, S. P., & Barros-Velázquez, J. (2012). Novel technologies for the preservation of chilled aquatic food products. In A. McElhatton, & P. Amaral Sobral (Eds.), *Novel Technologies in Food Science* (pp. 299–323). New York, USA: Springer (chapter 13).

Carissimi, M., Flôres, S., & Rech, R. (2018). Effect of microalgae addition on active biodegradable starch film. *Algal Research, 32,* 201-209.

Cérantola, S., Breton, F., Gall, E., & Deslandes, E. (2006). Co-occurrence and antioxidant activities of fucol and fucophlorethol classes of polymeric phenols in *Fucus spiralis*. *Botanica Marina, 49,* 347–351.

Chapman, R., & McKay, J. (1949). The estimation of peroxides in fats and oils by the ferric thiocyanate method. *Journal of the American Oil Chemists' Society, 26*, 360–363.

Cuter, C. N. (2006). Opportunities for bio-based packaging technologies to improve the quality and safety of fresh and further processed muscle foods. *Meat Science, 74,* 131–142.

Dehghani, S., Hosseini, S. V., & Regenstein, J. M. (2018). Edible films and coatings in seafood preservation: A review. *Food Chemistry, 240*, 505–513.

Etxabide, A., Uranga, J., Guerrero, P., & De la Caba, K. (2017). Development of active gelatin films by means of valorisation of food processing waste. *Food Hydrocolloids, 68*, 192–198.

Farvin, K., & Jacobsen, C. (2013). Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chemistry, 138*, 1670–1681.

Feng, X., Ng, V. K., Mikš-Krajnik, M., & Yang, H. (2017). Effects of fish gelatin and tea polyphenol coating on the spoilage and degradation of myofibril in fish fillet during cold storage. *Food and Bioprocess Technology, 10,* 89–102.

García-Soto, B., Aubourg, S. P., Calo-Mata, P., & Barros-Velázquez, J. (2013). Extension of the shelf-life of chilled-hake *(Merluccius merluccius*) by a novel icing medium containing natural organic acids. *Food Control, 34*, 356–363.

García-Soto, B., Miranda, J. M., Rodríguez-Bernaldo de Quirós, A., Sendón, R., Rodríguez-Martínez, A., Barros-Velázquez, J., & Aubourg, S. P. (2015). Effect of biodegradable film (lyophilised alga *Fucus spiralis* and sorbic acid) on quality properties of refrigerated megrim (*Lepidorhombus whiffiagonis*). *International Journal of Food Science and Technology, 50*, 1891–1900.

Giménez, B., López de Lacey, A., Pérez-Santín, E., López-Caballero, M. E., & Montero, P. (2013). Release of active compounds from agar and agar gelatin films with green tea extract. *Food Hydrocolloids, 30*, 264–271.

Goettems Kuntzler, S., Araujo de Almeida, A., Vieira Costa, J., & Greque de Morais, M. (2018). Polyhydroxybutyrate and phenolic compounds microalgae electrospun nanofibers: A novel nanomaterial with antibacterial activity. *International Journal of Biological Macromolecules, 113,* 1008–1014.

Gómez-Estaca, J., Montero, P., Giménez, B., & Gómez-Guillén, M. C. (2007). Effect of functional edible films and high pressure processing on microbial and oxidative spoilage in cold-smoked sardine (*Sardina pilchardus*). *Food Chemistry, 105*, 511–520.

Gupta, S., & Abu-Ghannam, N. (2011). Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science and Technology, 22*, 315–326.

Jo, W., Song, N., Lee, J., & Song, K. (2014). Physical properties and antimicrobial activities of a persimmon peel/red algae composite film containing grapefruit seed extract. *Food Science and Biotechnology, 23,* 1169–1172.

Kim, S., Back, S., & Song, K. (2018). Physical and antioxidant properties of alginate films prepared from *Sargassum fulvellum* with black chokeberry extract. *Food Packaging and Shelf Life, 18,* 157–163.

Kuda, T., & Ikemori, T. (2009). Minerals, polysaccharides and antioxidant properties of aqueous solutions obtained from macroalgal beach-casts in the Noto Peninsula, Ishikawa, Japan. *Food Chemistry, 112*, 575-581.

Kuley, E., Özoğul, F., & Polat, A. (2020). Advances in packaging. In Özoğul, Y. (Ed.), *Innovative Technologies in Seafood Processing* (pp. 45–69). Boca Raton, FL, USA: CRC Press, Taylor & Francis Group (chapter 3).

Lowry, R., & Tinsley, I. (1976). Rapid colorimetric determination of free fatty acids. *Journal of the American Oil Chemists' Society*, *53*, 470–472.

Mihindukulasuriya, S. D. F., & Lim, L. T. (2014). Nanotechnology development in food packaging. *Trends in Food Science and Technology, 40*, 149–167.

Min, B. J., & Oh, J. H. (2009). Antimicrobial activity of catfish gelatin coating containing origanum (*Thymus capitatus*) oil against Gram-negative pathogenic bacteria. *Journal of Food Science, 74*, 143–148.

Miranda, J. M., Trigo, M., Barros-Velázquez, J., & Aubourg, S. P. (2016). Effect of an icing medium containing the alga *Fucus spiralis* on the microbiological activity and lipid oxidation in chilled megrim (*Lepidorhombus whiffiagonis*). *Food Control*, *59*, 290–297.

Ólafsdóttir, G., & Jónsdóttir, R. (2010). Volatile aroma compounds in fish. In L. Nollet, & F. Toldrá (Eds.), *Handbook of Seafood and Seafood Products Analysis* (pp. 97–117). Boca Raton, Fl, USA: CRC Press.

Özoğul, Y. (2010). Methods for freshness quality and deterioration. In L. Nollet, & F. Toldrá (Eds.), *Handbook of Seafood and Seafood Products Analysis* (pp. 189–214). Boca Raton, Fl, USA: CRC Press, Taylor & Francis Group (chapter 13).

Paiva, L., Lima, E., Ferreira Patarra, R., Neto, A., & Baptista, J. (2014). Edible Azorean macroalgae as source of rich nutrients with impact on human health. *Food Chemistry*, *164*, 128–135.

Pavli, F., Tassou, C., Nychas, E. G.-J., & Chorianopoulos, N. (2018). Probiotic incorporation in edible films and coatings: Bioactive solution for functional foods. *International Journal of Molecular Sciences*, *19(1)*, 150.

Peinado, I., Girón, J., Koutsidis, G., & Ames J. M. (2014). Chemical composition, antioxidant activity and sensory evaluation of five different species of brown edible seaweeds. *Food Research International*, *66*, 36–44.

Pereira, L., Amado, A., Critchley, A., Van de Velde, F., & Ribeiro-Claro, P. (2009). Identification of selected seaweed polysaccharides (phycocolloides) by vibrational spectroscopy (FTIR-ATR and FT-Raman). *Food Hydrocolloids, 23,* 1903–1909.

Pokorný, J. (1981). Browning from lipid-protein interactions. *Progress in Food and Nutrition Science*, *5*, 421–428.

Rodríguez, O., Losada, V., Aubourg, S. P., & Barros-Velázquez, J. (2005). Sensory, microbial and chemical effects of a slurry ice system on horse mackerel (*Trachurus trachurus*). *Journal of the Science of Food and Agriculture, 85*, 235–242.

Rustad, T. (2010). Lipid oxidation. In L. Nollet, & F. Toldrá (Eds.), *Handbook of Seafood and Seafood Products Analysis* (pp. 87–95). Boca Raton, Fl, USA: CRC Press.

Sandsdalen, E., Haug, T., Stensvag, K., & Styrvold, O. (2003). The antibacterial effect of a polyhydroxylated fucophlorethol from the marine brown alga, *Fucus vesiculosus*. *World Journal of Microbiology and Biotechnology*, *19*, 777–782.

Sanjuás-Rey, M., García-Soto, B., Fuertes-Gamundi, J. R., Aubourg, S. P., & Barros-Velázquez, J. (2012). Effect of a natural organic acid-icing system on the microbiological quality of commercially relevant chilled fish species. *LWT-Food Science and Technology, 46*, 217–223.

Serrano, J., Puupponen-Pimia, R., Dauer, A., Aura, A., & Saura-Calixto, F. (2009). Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition and Food Research, 53*, S310–S329.

Sikorski, Z., & Kolakowski, E. (2000). Endogenous enzyme activity and seafood quality: Influence of chilling, freezing, and other environmental factors. In N. Haard, & B. Simpson (Eds.), *Seafood Enzymes* (pp. 451–487). New York, USA: Marcel Dekker.

Smit, A. (2004) Medicinal and pharmaceutical uses of seaweed natural products: A Review. *Journal of Applied Phycology, 16,* 245–262.

Stejskal, N., Miranda, J. M., Martucci, J. F., Ruseckaite, R. A., Aubourg, S. P., & Barros-Velázquez, J. (2020b). The effect of gelatine packaging film containing a *Spirulina platensis* protein concentrate on Atlantic mackerel shelf life. *Molecules, 25*, 3209.

Stejskal, N., Miranda, J. M., Martucci, J. F., Ruseckaite, R. A., Barros-Velázquez, J., & Aubourg, S. P. (2020a). Quality enhancement of refrigerated hake muscle by active packaging with a protein concentrate from *Spirulina platensis*. *Food and Bioprocess Technology, 13*, 1110–1118.

Taghavi Takyar, M. B., Haghighat Khajavi, S., & Safari, R. (2019). Evaluation of antioxidant properties of *Chlorella vulgaris* and *Spirulina platensis* and their application in order to extend the shelf life of rainbow trout (*Oncorhynchus* *mykiss*) fillets during refrigerated storage. *LWT-Food Science and Technology, 100*, 244–249.

Tierney, M., Smyth, T., Hayes, M., Soler-Vila, A., Croft, A., & Brunton, N. (2013a). Influence of pressurised liquid extraction and solid-liquid extraction methods on the phenolic content and antioxidant activities of Irish macroalgae. *International Journal of Food Science and Technology, 48*, 860–869.

Tierney, M., Smyth, T., Rai, D., Soler-Vila, A., Croft, A., & Brunton, N. (2013b). Enrichment of phenol contents and antioxidant activity of Irish brown macroalgae using food-friendly techniques based on polarity and molecular size. *Food Chemistry, 139*, 753–761.

Tilami, S. K., & Sampels, S. (2018). Nutritional value of fish: lipids, proteins, vitamins, and minerals. *Reviews in Fisheries Science, 26,* 242–253.

Umaraw, P., Munekata, P., Verma, A., Barba, F. J., Singh, V. P., Kumar, P., & Lorenzo, J. M. (2020). Edible films/coating with tailored properties for active packaging of meat, fish and derived products. *Trends in Food Science and Technology, 98*, 10–24.

Vyncke, W. (1970). Direct determination of the thiobarbituric acid value in trichloracetic acid extracts of fish as a measure of oxidative rancidity. *Fette, Seifen, Anstrichmittel, 72,* 1084–1087.

FIGURE LEGENDS

Figure 1: Development of aerobe counts (log CFU·g^{-1} muscle)* in refrigerated mackerel stored under different packaging conditions**

* Average values of three replicates (*n*=3); standard deviations are indicated in bars. For each refrigeration time, values accompanied by different lowercase letters denote significant differences (*p*<0.05) as a result of the packaging system. For each packaging system, values accompanied by capital letters denote significant differences (*p*<0.05) as a result of the refrigeration time.

** Packaging conditions: CT (control packaging), F-1 (lowest-concentrated alga film packaging), and F-2 (highest-concentrated alga film packaging).

Figure 2: Development of psychrotroph counts (log CFU·g^{-1} muscle)* in refrigerated mackerel stored under different packaging conditions**

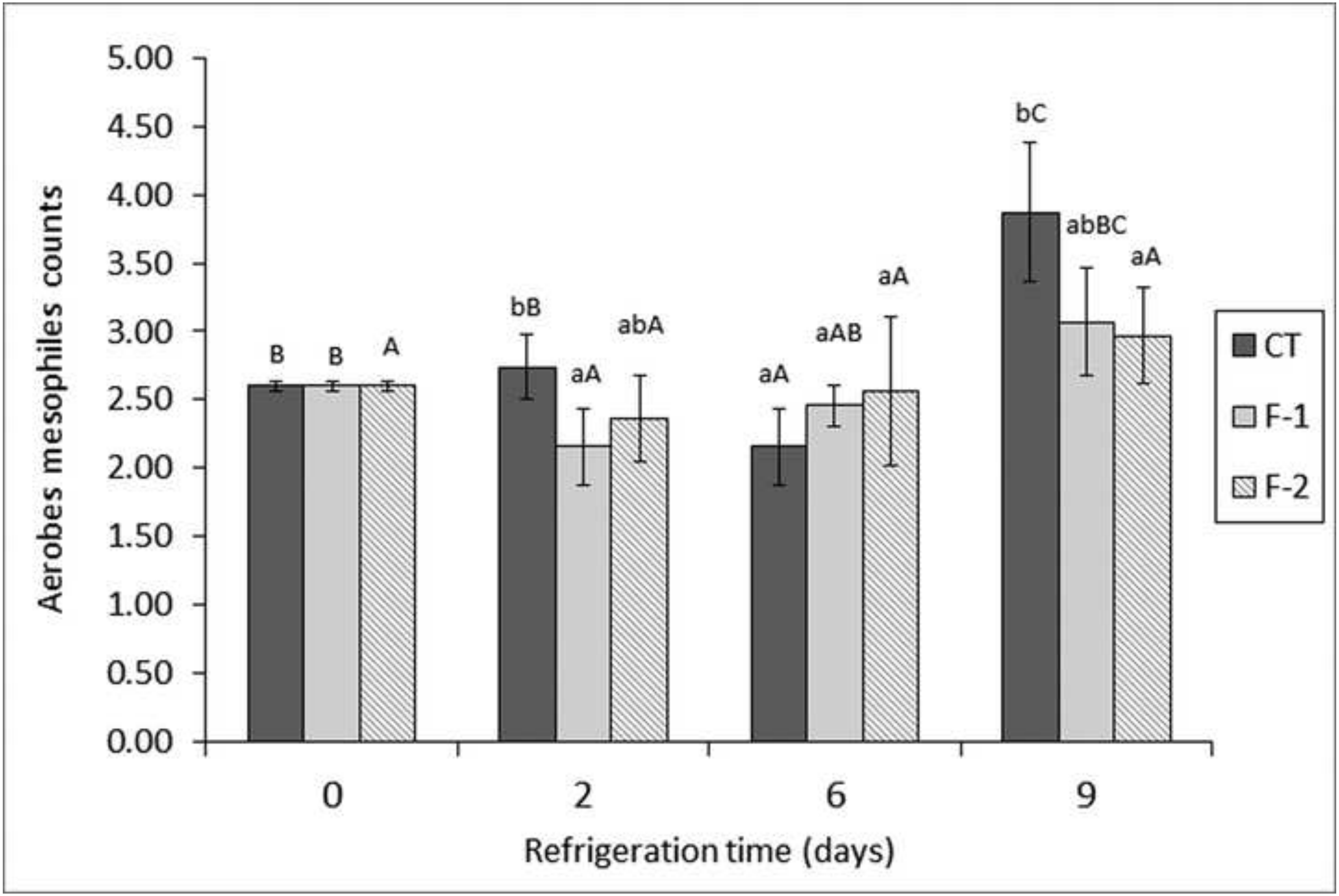
* Average values of three replicates (*n*=3); standard deviations are indicated in bars. For each refrigeration time, values accompanied by different lowercase letters denote significant differences (*p*<0.05) as a result of the packaging system. For each packaging system, values accompanied by capital letters denote significant differences (*p*<0.05) as a result of the refrigeration time.

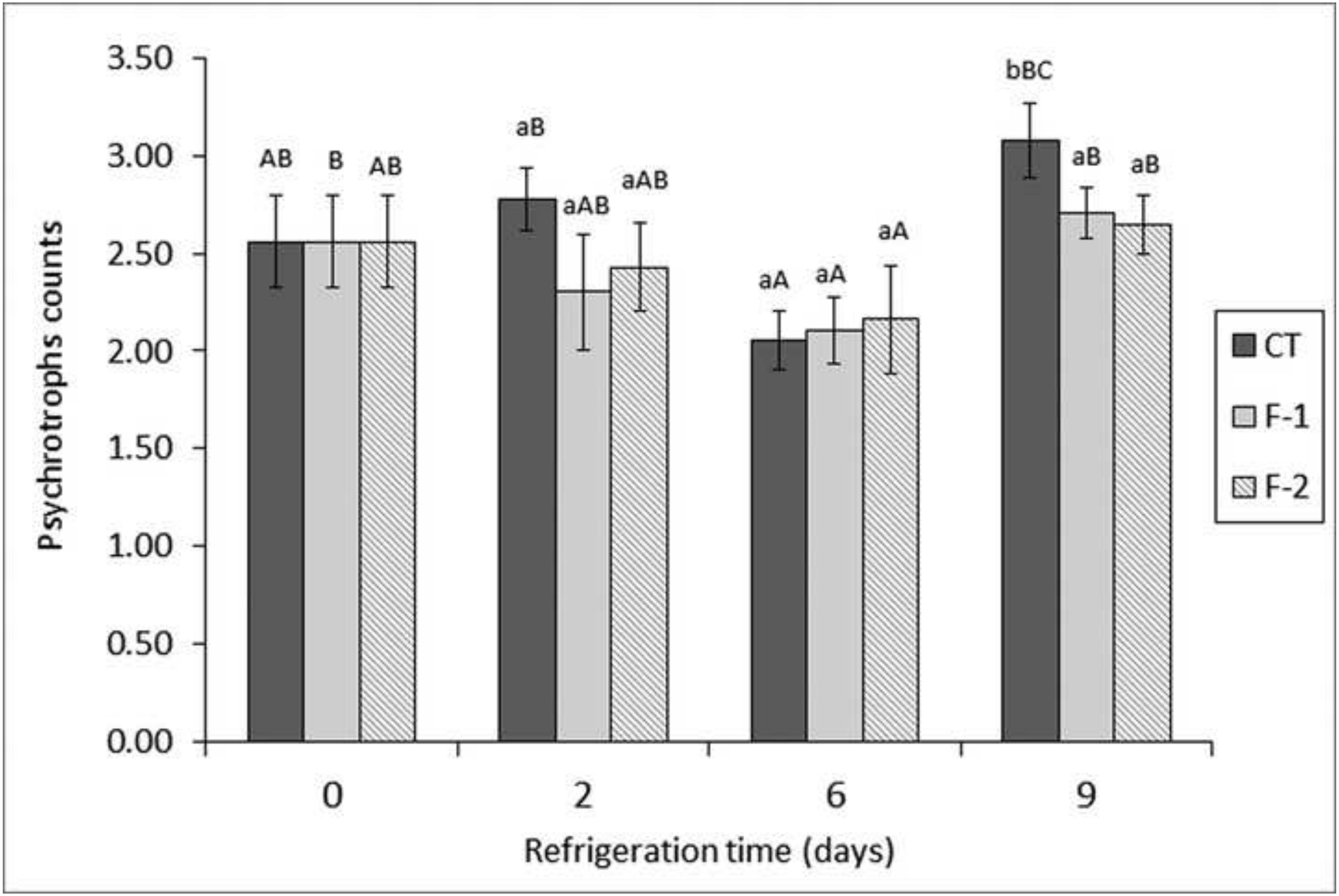
** Packaging systems as indicated in Figure 1.

Figure 3: Determination of free fatty acid (FFA) content (mg·kg^{-1} muscle)* in refrigerated mackerel stored under different packaging conditions**

* Average values of three replicates (*n*=3); standard deviations are indicated in bars. For each refrigeration time, values accompanied by different lowercase letters denote significant differences ($p<0.05$) as a result of the packaging system. For each packaging system, values accompanied by capital letters denote significant differences ($p<0.05$) as a result of the refrigeration time.

** Packaging conditions as indicated in Figure 1.


Aerobes mesophiles counts
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
B
B
A
bB
aA
abA
aA
aAB
aA
bC
abBC
aA
0
2
6
9
Refrigeration time (days)
CT
F-1
F-2


Psychrotrophs counts
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
AB
B
AB
aB
aAB
aAB
aA
aA
aA
bBC
aB
aB
0
2
6
9
Refrigeration time (days)
CT
F-1
F-2

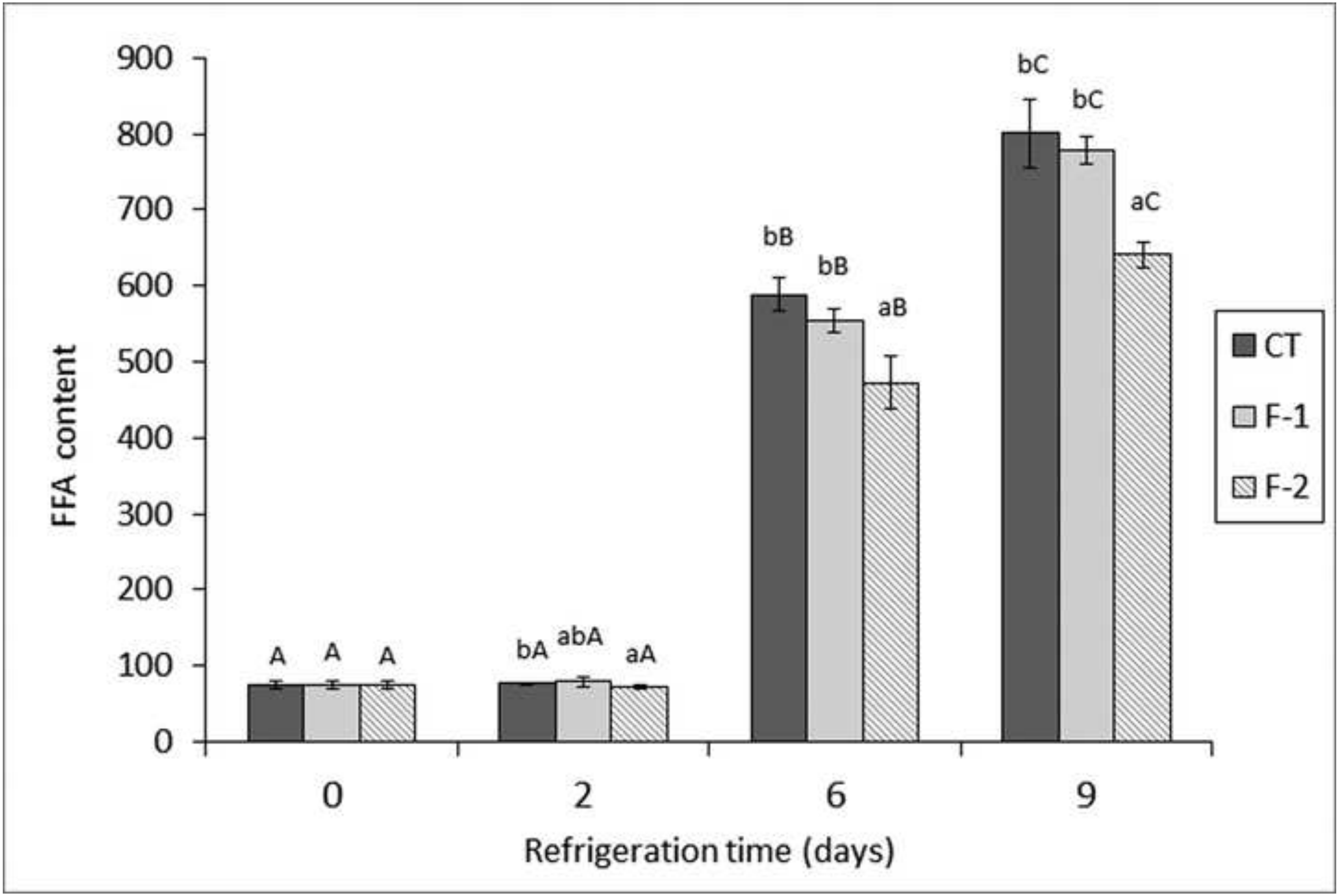

FFA content
900
800
700
600
500
400
300
200
100
0
A
A
A
bA
abA
aA
bB
bB
aB
bC
bC
aC
0
2
6
9
Refrigeration time (days)
CT
F-1
F-2

TABLE 1

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

Microbial group	Packaging condition	Refrigeration time (days)			
		0	2	6	9
Proteolytics	CT	2.00 A (0.02)	2.16 aA (0.28)	2.05 aA (0.03)	3.53 bB (0.68)
	F-1	2.00 A (0.02)	2.33 aAB (0.58)	2.07 aA (0.06)	2.72 aB (0.10)
	F-2	2.00 A (0.02)	2.10 aA (0.17)	2.02 aA (0.06)	3.15 abB (0.83)
Lipolytics	CT	2.00 A (0.01)	2.00 aA (0.00)	2.00 aA (0.00)	3.13 aB (0.31)
	F-1	2.00 A (0.01)	2.00 aA (0.00)	2.00 aA (0.00)	2.72 aB (0.10)
	F-2	2.00 A (0.01)	2.00 aA (0.00)	2.00 aA (0.00)	2.74 aB (0.13)
Anaerobes	CT	2.62 A (0.28)	2.42 aA (0.39)	2.36 aA (0.39)	2.92 aA (0.35)
	F-1	2.62 A (0.28)	2.39 aA (0.35)	2.30 aA (0.30)	2.60 aA (0.21)
	F-2	2.62 A (0.28)	2.30 aA (0.10)	2.46 aA (0.56)	2.52 aA (0.24)

* Average values of three replicates (*n*=3); standard deviations are indicated in brackets. In each column, values accompanied by different low-case letters denote significant differences (*p*<0.05) as a result of packaging. In each row, values accompanied by capital letters denote significant differences (*p*<0.05) as a result of refrigeration time.

** Packaging conditions: CT (control packaging), F-1 (lowest-concentrated alga packaging) and F-2 (highest-concentrated alga packaging).

TABLE 2

Determination of lipid oxidation* in refrigerated mackerel stored under different packaging conditions**

Chemical index	Packaging condition	Refrigeration time (days)			
		0	2	6	9
Peroxide value (meq active oxygen·kg^{-1} lipids)	CT	1.95 A (0.78)	2.34 aA (0.41)	7.24 aB (1.40)	11.82 aC (0.93)
	F-1	1.95 A (0.78)	1.31 aA (0.63)	7.98 aB (1.33)	11.98 aC (1.24)
	F-2	1.95 A (0.78)	2.44 aA (0.45)	11.05 bB (0.88)	12.32 aB (2.13)
Thiobarbituric acid index (mg malondialde-hyde·kg^{-1} muscle)	CT	0.03 A (0.03)	0.31 aB (0.08)	2.59 aC (0.28)	3.65 aD (0.70)
	F-1	0.03 A (0.03)	0.55 bB (0.03)	3.11 abC (1.03)	5.76 bD (1.12)
	F-2	0.03 A (0.03)	0.86 cB (0.20)	3.81 bC (0.47)	8.64 cD (1.01)
Fluorescence ratio	CT	2.34 A (0.90)	11.14 bB (1.10)	14.19 bBC (2.11)	16.86 bC (1.71)
	F-1	2.34 A (0.90)	6.31 aB (0.94)	13.82 bC (3.72)	16.45 bC (3.27)
	F-2	2.34 A (0.90)	5.82 aB (0.57)	6.45 aB (1.52)	9.48 aC (1.03)

* Average values of three replicates (*n*=3); standard deviations are indicated in brackets. In each column, values accompanied by different low-case letters denote significant differences ($p<0.05$) as a result of packaging. In each row, values accompanied by capital letters denote significant differences ($p<0.05$) as a result of refrigeration time.

** Packaging conditions as expressed in Table 1.

Conflict of interest

The authors declare no conflict of interest.

CREDIT AUTHOR STATEMENT

Author contributions:

Conceptualization (SPA and JBV), methodology (MT, PN, and JMM), data curation (MT, PN, JMM, and SPA), writing-original draft (SPA and JBV) and writing-review and editing (SPA and JBV).

All authors have read and agreed with the revised version.