Food Packaging and Shelf Life 25 (2020) 100522

Contents lists available at ScienceDirect

Food Packaging and Shelf Life

journal homepage: www.elsevier.com/locate/fpsl

A novel method to deliver natural antimicrobial coating materials to extend the shelf-life of European hake (*Merluccius merluccius)* fillets

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ARTICLE INFO

Keywords:
Aerosolisation
Natural antimicrobials
Nanomaterials
Coating
Hake fillets (Merluccius merluccius)
Shelf-life

ABSTRACT

The objectives of this study were to investigate the effectiveness of aerosolisation as a novel method for coating natural antimicrobials solutions of chitosan (CS), chitosan nanoparticles (CS NP) and commercially available carnosolic acid nano-solubilisate (CASB) with a view to provide an additional hurdle to extend the shelf-life of vacuum skin packaging (VSP) European Hake (*Merluccius merluccius*) fillets. The aerosolisation of hake fillets with natural antimicrobial solutions of 0.1 % w/v CS, 0.1 % w/v CS NP, or 0.8 % w/v CASB extended significantly ($P < 0.05$) the shelf-life of VSP hake fillets by up to 40, 50, and 55 %, respectively compared to untreated control samples. Moreover, the pH of hake fillets was significantly ($P < 0.05$) increased by CS and CS NP treatments compared to untreated control and CASB treatments. The colour parameters of treated hake fillets increased significantly throughout storage, independent of antimicrobial treatment for all samples. However, lightness was significantly ($P < 0.05$) reduced by CASB treatments (compared to other treatments), redness was significantly ($P < 0.05$) increased by CS NP treatments (compared to other treatments), and yellowness was significantly ($P < 0.05$) increased with all CS, CS NP, and CASB treatments (compared to control samples). In addition, the whiteness index (WI) of hake fillets was reduced significantly ($P < 0.05$) when aerosolised with CS, CS NP, and CASB treatments. The lipid oxidation (TBARS) of hake fillets, independent of the antimicrobial treatment, decreased significantly ($P < 0.05$) throughout storage. The results from this study indicate that aerosolisation of natural antimicrobial materials can effectively extend the shelf-life of fish products.

1. Introduction

Hake fillets (*Merluccius merluccius*) are a high-value food product, mostly consumed in European markets. However, hake, like many other fish products are highly perishable and deteriorate rapidly post mortem due to various biochemical and microbial breakdown mechanisms as a consequence of their high water content (65 – 80 %), mild pH (6 – 7) as well as the presence of large quantities of non-protein nitrogen (9 - 18 %). Additionally, environmental factors such as the catching region, season, and handling conditions post mortem all contribute to the short shelf-life of hake fillets (Fernandez-Saiz, Sanchez, Soler, Lagaron, & Ocio, 2013; Garcia-Soto, Aubourg, Calo-Mata, & Barros-Velazquez, 2013; Otero, Perez-Mateos, & Lopez-Caballero, 2017) and it is estimated that 30 – 50 % of all harvested fish are lost in the supply chain (Garcia et al., 2015).

Typically, fish products intended for consumers are packaged using technologies such as modified atmosphere packaging (MAP), vacuum packing (VP) and vacuum-skin packaging (VSP). It was reported that compared to aerobically stored hake, these packaging technologies have considerably extended the shelf-life of hake fillets (Carrion-Granda, Fernandez-Pan, Rovira, & Mate, 2018; Erkan, 2012). Moreover, further shelf-life extension can be achieved through the application of hurdle technology, which is an intelligent combination of mild preservation technologies (Kerry, O'Grady, & Hogan, 2006). One such hurdle is the application of natural antimicrobial materials (NAM's) such as chitosan (CS) and essential oil (EO) based materials. The use of these materials is viewed as favourable due to their properties such as: biodegradability, biocompatibility, "GRAS" status and antimicrobial properties against a wide range of spoilage and pathogenic microorganisms (Sullivan, Azlin-Hasim et al., 2018). Currently, NAM's are

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https://doi.org/10.1016/j.fpsl.2020.100522
Received 17 April 2019; Received in revised form 28 March 2020; Accepted 19 May 2020
Available online 08 June 2020

applied to food products as part of the hurdle approach through various technologies including: direct incorporation into the polymeric packaging material (Yang et al., 2016), coating onto packaging materials surface (Erkan, 2012), and using an inherently antimicrobial film forming polymer (such as CS) (Fernandez-Saiz et al., 2013) or a combination thereof (Shahbazi & Shavisi, 2018). These strategies, in combination with NAMs, have been used to extend the shelf-life of a wide variety of food products such as; tomatoes (Tripathi, Mehrotra, & Dutta, 2009), chicken breasts (Latou, Mexis, Badeka, Kontakos, & Kontominas, 2014), sea bass fillets (Günlü & Koyun, 2013), and ready-to-eat (RTE) shrimp (Guo, Jin, Scullen, & Sommers, 2013), oysters (Rong, Qi, Yin, & Zhu, 2010), salmon (Souza et al., 2010), silver carp (Abdollahi, Rezaei, & Farzi, 2014), cod fillets (Gomez-Estaca, Lopez de Lacey, Lopez-Caballero, Gomez-Guillen, & Montero, 2010), whiteleg shrimp (Wang et al., 2015), and beef (Vilela et al., 2016).

Aerosolisation is defined as the dispersion of a liquid phase into air in the form of a fine mist and is usually used for sanitary purposes, especially for respiratory medical treatments (Andersen et al., 2006). However, to the best of our knowledge the application of aerosolised NAM's to fish products as a novel method to deliver antimicrobial coating and enhance microbiological quality and shelf life extension of these products has not been investigated. This type of delivery system offers numerous advantages including: relative ease of application, higher surface coverage, and greater antimicrobial penetration due to the small droplet compared to conventional spray coating size. Nonetheless, minor drawbacks include the scaling up of the aerosolisation unit for commercial application and the limited number of antimicrobials that can be aerosolised (Jiang et al., 2017). Nonetheless, nebulizers are a readily available apparatus that are widely used as an aerosolisation system for delivery of drugs into the lungs. This is via the generation of a fine "mist" with a typical average droplet diameter of ≥ 5 μm. This is a smaller droplet diameter than conventional spray coated generated mist or from a typical electrostatic sprayer (Jiang et al., 2017; Park et al., 2012). Currently, most studies involving aerosolisation treatment systems have focused on organic acid sanitisers and disinfectants such as peroxyacetic acid and sodium hypochlorite due to their water solubility and broad antimicrobial activity and have been applied on to various fruits such as cherry tomatoes (Jiang et al., 2017) and strawberries (Vardar, Ilhan, & Karabulut, 2012). However, nanotechnology, which is the development and application of materials that have one or more dimensions of the order of 100 nanometres (nm) or less (Chaudhry et al., 2008), may allow for the increase in scope of materials that could be applied via aerosolisation due to their small size features which could be readily dispersed, either as a particle, colloid or micelle, in an aerosolisable aqueous solution. Accordingly, the use of the nano equivalents of CS and EO's have been widely reported as antimicrobial active agent in food packaging applications (Sullivan, Azlin-Hasim et al., 2018), and given their previously outlined properties make them ideally suited to this application.

While studies have reported on the use of aerosolisation of antimicrobials as a treatment method to extend shelf of food products (Jiang et al., 2017; Vardar et al., 2012); to the best of our knowledge, none have investigated the use of aerosolisation of nano and non-nano NAM as means to deliver antimicrobial coating as treatment in a hurdle strategy to extend the shelf-life of hake fillets. Therefore, the objective of this study was to evaluate the effects of the aerosolisation of CS, CS NPs and CASB antimicrobial solutions, as an additional hurdle to extend the shelf life of VSP hake fillets.

2. Materials and Methods

2.1. Materials

Skinned Hake fillets (hitherto referred to as hake fillets) caught in the Celtic Sea (FAO 27) were purchased from Ballycotton Seafood Ltd (Garryvoe, Ireland). Low molecular weight chitosan (L. MW) (MW; 50 – 190 kDa, ≥75% deacetylated), aqueous acetic acid (HOAc), sodium hydroxide (NaOH) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. NovaSOL® Rosemary, a commercially available novel water and fat soluble 6% carnosolic acid nano-solubilisate (CASB) was obtained from AQUANOVA ® (AQUANOVA AG, Darmstadt, Germany). As per the manufacturer specification sheet, Novasol® Rosemary was composed of polysorbate 80 (E433), polysorbate 20 (E432), and rosemary extract containing a minimum of 45 % Carnsolic acid. The pH of the NovaSOL® solution is between pH 4 – 7. Media for microbiological analysis including; plate count agar, De Man, Rogosa and Sharpe (MRS) agar and *Pseudomonas* Agar Base with selective supplement CFC (cetrimide, fucidin, cephaloridine) (SR0103) were purchased from Oxoid, while Trytic Soy Agar was purchased from Merck. Compact Dry-EC chromogenic plates were obtained from Nissui Pharmaceutical (Co. Ltd. Japan). Lyngby agar was made using (g L^{-1}); peptone (20), bacteriological agar (15), sodium chloride (5), sodium thiosulfate (0.3), yeast extract (3), beef extract (3), L-cysteine (0.6) and ferric citrate (0.3) which were all purchased from Sigma Aldrich.

2.2. Preparation and aerosolisation of antimicrobials onto Hake fillets

2.2.1. Preparation of natural antimicrobial solutions

Solutions of 0.1 % L. MW CS and CS NP were prepared as previously outlined by (Sullivan, Cruz-Romero et al., 2018). Briefly, 0.1 % CS solutions were prepared by dissolving CS in a 1 % v/v aqueous acetic acid solution and then the pH adjusted to 4.6. CS NPs were prepared by dissolving L. MW CS into 1 % v/v aqueous acetic acid solution whereupon TPP was added in a 3:1 CS;TPP ratio and subsequently the pH of the solution was adjusted to 4.6 using 1 M NaOH. For the nanosolubilisate, the 6 % CASB was heated to 40 °C prior to diluting with sterile distilled water to a final concentration of 0.8% of the active component (carnosolic acid) as this was the highest aerosolisable concentration.

2.2.2. Preparation, aerosolisation and vacuum skin packaging of Hake fillet samples

Using a sterile knife, hake fillets were cut into ca. 150 g portions and then four pieces of the portioned fish were individually placed on a sterilized wire rack that was placed inside a specially modified 7 L container (Sistema, New Zealand) to hold the fish fillets and allow the permeation of the aerosolised antimicrobials on the surface of the hake fillets (Fig. 1.). The lid of the container was tightly closed and 12 mL (of which 10.5 mL was aerosolised onto the Hake fillets) of 0.1 % w/v CS, 0.1 % w/v CS NP or 0.8 % CASB antimicrobial solutions were loaded into the receiver cup of the atomiser nebuliser (3A, Lonato del Garda, Italy) and aerosolised for 30 min at a rate of 0.35 mL min^{-1}. The aerosolised hake fillets were then removed aseptically from the chamber, and individually placed onto a recyclable polyethylene terephalate (rPET/PE) tray (590 mm × 390 mm × 475 mm; ES Plastic GmbH & Co KG, Germany) and then vacuum skin packed with a coextruded SkinFreshTop 80 PE/EVOH/PE film (oxygen permeability of < 5 cm^3 m^{-2} dbar) using an ILPRA FP Basic VG machine (Vigevano, Italy). VSP hake fillets were stored at 4 °C until use and sampling was carried out every three days and for each sampling day, two trays were randomly selected for microbiological and physicochemical analysis. The whole experiment was repeated independently three times and results are the average of 6 replicates.

2.3. Physiochemical Analysis

2.3.1. Proximal Analysis

Fat and moisture of hake fillets were determined using the CEM Analysis System (CEM Corporation, Matthews, NC 28105, USA) (Bostian, Fish, Webb, & Arey, 1985); protein content was determined according to AOAC Procedures (1999) (method 981.10). The ash

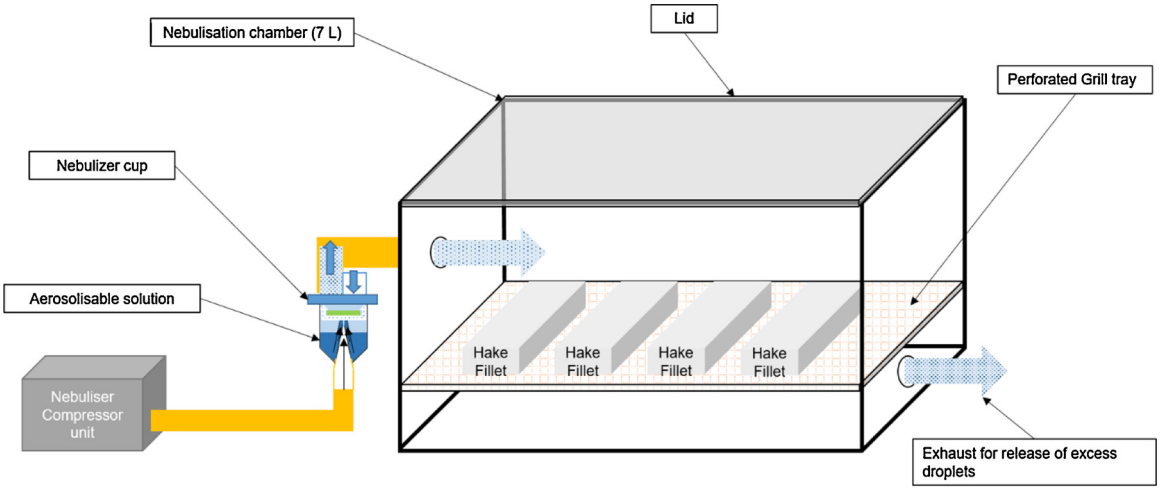

Fig. 1. Schematic set up of the aerosolisation apparatus.

content of the hake fillets were determined by incineration of the hake samples in a furnace (Nabertherm, Model L9/C6, Nabertherm, Germany) at 550 °C.

2.3.2. pH

The pH of the hake fillets were measured using a previously calibrated digital pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) by inserting the glass probe directly into the hake flesh. Each value represents the average of 7 measurements.

2.3.3. Colour

The surface colour of hake fillets during chilled storage at 4 °C was recorded using a Minolta chromameter (CR-300, Minolta Camera Co., Osaka, Japan) as outlined by (Azlin-Hasim, Cruz-Romero, Morris, Cummins, & Kerry, 2015). Briefly, 10 random areas of the hake surface were measured per sample on each measurement day and the average values of ten readings of CIE *L*-value (lightness), *a*-value (redness) and *b*-value (yellowness) were recorded. Whitening index (WI) was calculated using Eq. (1) (Borchert et al., 2014):

$$WI = L - (3b) + (3a) \quad (1)$$

2.3.4. Lipid oxidation

The lipid oxidation of hake fillets were assessed by measuring the 2-thiobarbituric acid-reactive substances (TBARS) assay as outlined by (Siu & Draper, 1978) and the malondialdehyde (MDA) content was calculated using an extinction coefficient of 1.56×10^5 L mol^{-1} cm^{-1} and results were expressed in mg MDA kg^{-1} of hake fillets.

2.4. Microbiological analysis

To obtain a representative sample, 10 g of hake flesh was aseptically taken (5 g of hake flesh from the top side and 5 g from bottom side of the hake fillet) and placed in a sterile stomacher filter bag. Then, 90 ml of sterile MRD was added aseptically into a stomacher bag and was homogenised for 180 s using BA6021 stomacher 400 (Colworth, Bury St. Edmunds, UK). The homogenate was then ten-fold diluted and used for the enumeration of: total viable counts (TVC), psychrotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria (LAB), total coliforms and *Escherichia coli* (*E. coli*), anaerobic bacteria, and H_2S-producing bacteria. TVC and psychotropic bacteria analysis was enumerated on PCA using the pour plate method and incubated at 37 °C for 48 h or 4 °C for 1 week, respectively. Lactic acid bacteria (LAB) was enumerated on de Man, Rogosa and Sharpe (MRS) agar using a pour plate method with an overlay and incubated at 30 °C for 72 h. Anaerobic bacteria was enumerated using spread plate method on tryptic soy agar (TSA) containing 0.6 % yeast extract and incubated under anaerobic conditions in anaerobe jars with Anerocult A (Merck) for 72 h at 30 °C. H_2S-producing bacteria was enumerated on Lyngby iron agar after incubation at 30 °C for 72 h. *Pseudomonas* spp. was enumerated using spread plate method after 2 days incubation at 30 °C on *Pseudomonas* agar base to which CFC 0103 supplement was added. For the enumeration of total coliforms and *E. coli*, 1 ml of the appropriate dilution was placed in duplicate onto the centre of Compact Dry-EC chromogenic plates and incubated at 37 °C for 24 h. As indicated by the manufacturer, blue colonies were counted as *E. coli* and total coliforms included red and blue colonies.

2.5. Statistical analysis

All data was analysed for means and standard deviations, and analysis of variance. One-way analysis of variance of data was carried out using the SPSS 24 for Windows (SPSS Statistical software, IBM Corp., Armonk, NY, USA) software package. Differences between pairs of means was resolved by means of confidence intervals using Tukey's test; the level of significance was set at $P < 0.05$.

3. Results and discussion

3.1. Proximal Composition

The proximal composition of hake fillets caught in the Irish Sea indicated that hake fillets had 79.73, 17.57, 0.9, 0.26 and 1.07 % moisture, protein, fat and ash content, respectively (data not shown). These results were broadly in agreement with a study carried out by (Roncarati et al., 2012) who found that hake caught in the south Tyrrhenian sea had 79.9, 17.9, 1.2 and 1.54 % moisture, protein, lipid and ash content, respectively. The small variations in the composition of hake fillets occur due to environmental and physiological factors such as the nutrition, catching season (spawning cycles), sexual variation, fish size, living area, as well as the other environmental conditions (Dominguez-Petit, Saborido-Rey, & Medina, 2010). Furthermore, after aerosolisation treatment with the antimicrobial solutions, the proximal composition of hake fillets were found not to be affected significantly ($P > 0.05$) (data not shown); as only a thin layer of antimicrobial substance was deposited on the surface of the hake fillets which had a negligible effect on the proximal composition and, to the best of our knowledge, this has not been reported elsewhere as typically coatings can affect significantly the proximate composition of fish (Yıldız, 2017).

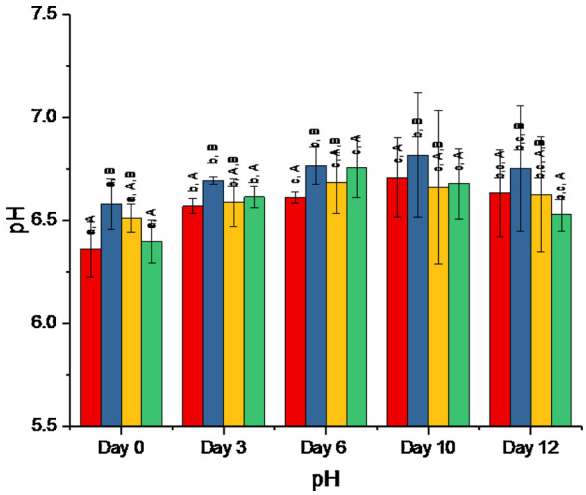

Fig. 2. Changes in pH of control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4 °C under vacuum skin packaging over the 12 day storage period. [a,b] Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and [A,B] with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

3.2. Changes on pH of Hake fillets during storage

The pH of control and antimicrobial treated VSP hake fillets stored at 4 °C are shown in Fig. 2. Independent of antimicrobial treatment, the pH of the hake fillets increased significantly ($P < 0.05$) throughout storage until day 6. Moreover, independent of the storage day, CS and CS NP treated hake fillets were found to increase the pH of hake fillets significantly ($P < 0.05$) compared to both untreated control and CASB treatments. The observed increase in the pH of hake fillets over time could be attributed to the accumulation of undesirable alkaline compounds, such as ammonia and trimethylamine (TMA) which arise from microbial action (Garcia-Soto et al., 2013). Nonetheless, results indicated that the hake fillets remained below pH 7, which is the upper limit for organoleptic acceptability (Volpe et al., 2015) and these results are in agreement with (Garcia-Soto et al., 2013) who observed no definite pH trend during storage time with respect to antimicrobial treatments.

3.3. Colour of Hake fillets during storage

The effect of storage time and antimicrobial treatments on the CIE L-value (lightness), a-value (redness), b-value (yellowness) and whiteness index (WI) of hake fillets are shown in Fig. 3. Independent of the antimicrobial treatment used, L-values were found to increase significantly ($P < 0.05$) until day 9, whereupon the lightness began to decrease. Independent of the storage time, Hake fillets treated with either CS, CS NP or CASB were observed to be significantly ($P < 0.05$) darker than untreated control hake fillets. The higher darkness of CS and CS NP treated hake fillets may perhaps be due to the low pH of the solutions used as CS and CS NP were dissolved in 1% acetic acid solution before aerosolisation treatment which may resulted in the leaching of muscle pigments (Mohan, Ravishankar, Lalitha, & Srinivasa Gopal, 2012). In relation to CASB, the dark brown/olive colour of the aerosolisable solution may perhaps be reducing the lightness of the hake fillet (Fig. 3 a.).

Regarding a-values, independent of the antimicrobial treatment used, the redness was observed to only begin decreasing significantly ($P < 0.05$) after day 9 (Fig. 3 b). However, independent of storage time, CS NPs treated hake fillets were found to significantly ($P < 0.05$) higher a-values compared to either the control, CS or CASB treated hake fillets and may perhaps be due to the strong chelating ability of CS NPs coordinating to pro-oxidant materials decreasing lipid oxidation (Yen, Yang, & Mau, 2008). Nonetheless, hake fillets have relatively low redness values due to naturally low concentration of "redness" increasing pigments such as carotenoids and/or hemopigments (Sanchez-Zapata, Perez-Alvarez, Fernandez-Lopez, & Barber, 2010).

Concerning b-values, a significant ($P < 0.05$) increase was observed up to day 9, independent of the antimicrobial treatment used (Fig. 3 c.). With respect to antimicrobial treatments, it was observed that untreated control hake differed significantly ($P < 0.05$) to CS, CS NP, or CASB treated hake fillets. The increase in b-values for control hake fillets may be associated with the increased oxidation and formation of metmyoglobin (Ghaderi-Ghahfarokhi, Barzegar, Sahari, & Azizi, 2016).

Regarding the WI, significant ($P < 0.05$) differences between control, CS, and CS NP or CASB treated hake fillets were observed (Fig. 3.d.). Overall, control treated hake fillets had the highest WI, while the lowest WI was obtained when hake fillets were aerosolised with CASB

3.4. TBARS of Hake fillets during storage

The lipid oxidation of control, CS, CS NP, and CASB treated hake fillets are shown in Fig. 4. It was observed that, independent of the antimicrobial treatment used, the TBARS values decreased significantly ($P < 0.05$). Moreover, untreated control hake fillets were found to have a higher TBARS than CS, CS NP or CASB treated hake fillets, this was not statistically significant. It was reported that the decrease in TBARS may be as a result of the reaction between malondialdehyde and components of the fish muscle such as proteins, amino acids and glycogen which will decrease the amount of malondialdehyde present (Shahbazi and Shavisi, 2018). Furthermore, the antimicrobials used here have all been reported to have antioxidant properties which may perhaps be "mopping up/reacting with" lipid oxidation initiators; therefore, reduce lipid oxidation pathways (Ojagh, Rezaei, Razavi, & Hosseini, 2010; Shahbazi & Shavisi, 2018; Yang et al., 2016). It has been reported that the antioxidant mechanism of action of CS is related to the metal-chelation ability of CS to form a complex with lipids and good scavenging ability on hydroxyl radicals and that the antioxidant properties are increased with the degree of N-deacetylation (Kim & Thomas, 2007). Conversely, CASB which is derived from rosemary essential oils, has a phenolic structure which have been shown to possess remarkable antioxidant activity due to their ability to capture chain-carrying lipid peroxyl radicals associated with lipid oxidation and quench their propagation through a radical-radical reaction (Amorati, Foti, & Valgimigli, 2013). In addition to the antimicrobial treatments used, VSP has been shown to reduce the effect of lipid oxidation, especially in comparison to MAP, due to the presence of CO_2 in MAP denaturing muscle proteins that liberates iron and can acts as pro-oxidant in the lipid fraction; however, in VSP there is negligible CO_2 to initiate lipid oxidation via this process (Perez-Alonso, Aubourg, Rodriguez, & Barros-Velazquez, 2004; Rodrigues et al., 2016). Nonetheless, throughout the storage period, the TBARS remained below the 1.5 mg kg^{-1} upper limit beyond which fish will normally develop objectionable odours/tastes (Cruz-Romero, Kelly, & Kerry, 2008). The results reported in this study are in agreement with the results reported by (Garcia-Soto et al., 2013) who found that lipid oxidation is not an important deterioration parameter during chilled storage of lean white fish species such as hake fillets.

3.5. Microbiological analysis of Hake Fillets

From a microbiological perspective, fresh fish with an aerobic TVC below 5×10^5 CFU g^{-1} are considered to be of good quality while the unacceptable limit is 7 log CFU g^{-1} (EC 2073/05, 2005EC 2073/05, 2005; ICMSF (International Commission on Microbiological Specifications for Foods), 1980). In this study, a marginal TVC value of

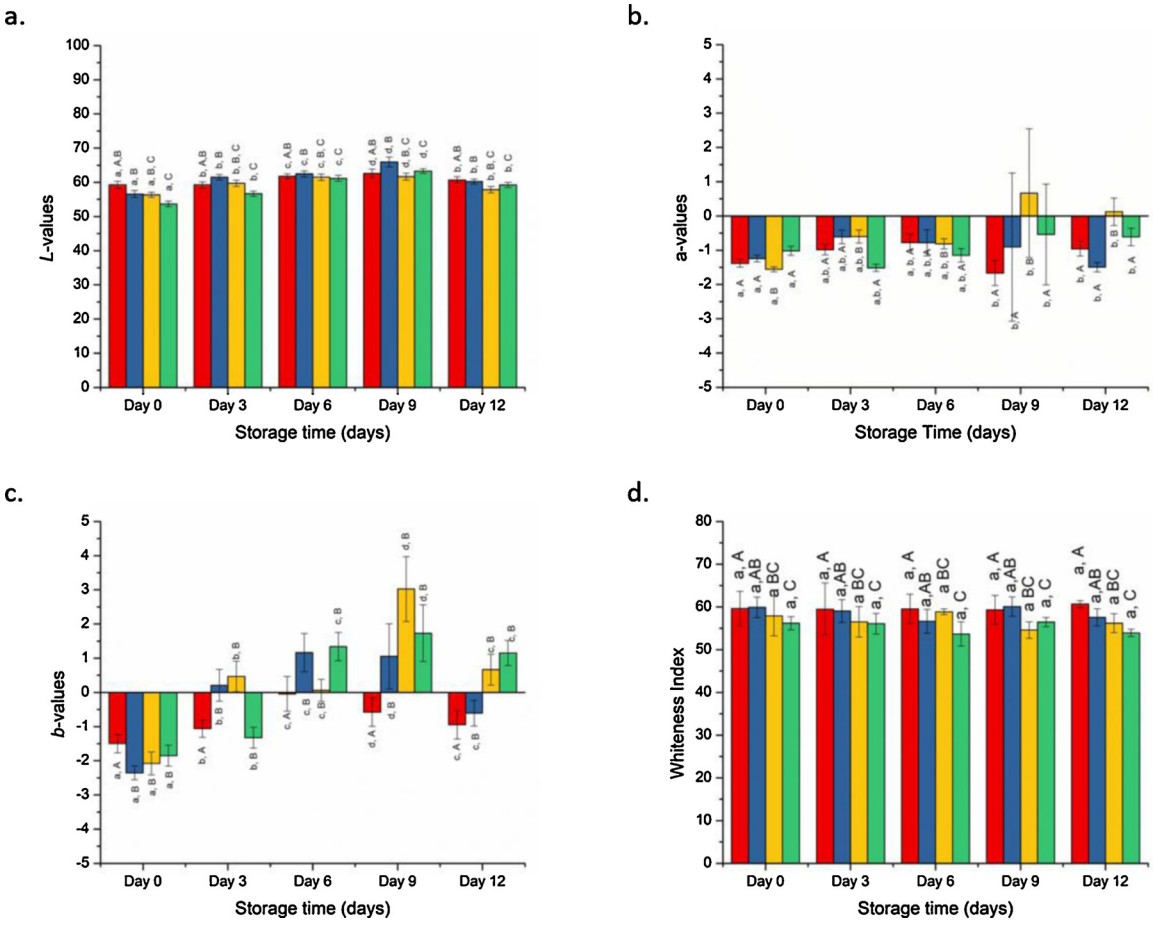

Fig. 3. Lightness (L-values) (a.), redness (a-values)(b.), yellowness (b-values) (c.), and whiteness index (d.) of control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4 °C under vacuum skin packaging over the 12 day storage period. [a,b] Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and [A,B] with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

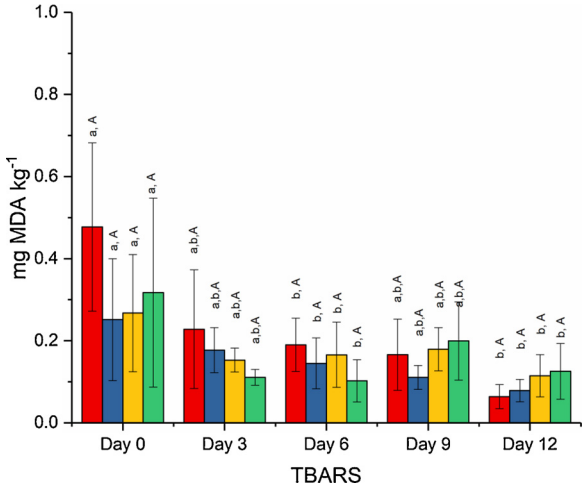

Fig. 4. Changes in TBAR'S (mg MDA kg^{-1}) of control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4 °C under vacuum skin packaging over the 12 day storage period. [a,b] Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and [A,B] with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

6 log $CFUg^{-1}$ was assigned as the maximum limit of acceptability. The effect of antimicrobial treatments in combination with VSP stored at 4 °C on the microbial counts of TVC, anaerobes, psychrotrophs, *Pseudomonas* spp., H_2S-producing bacteria, LAB, *E. coli,* and coliforms are shown in Fig. 5. Over the storage period, significantly ($P < 0.05$) lower bacterial population of TVC, anaerobic and coliform bacteria was observed in hake fillets that were treated with either CS, CS NP or CASB compared to the untreated control hake fillets. Similarly for LAB, *Pseudomonas* spp., psychrotrophs, *E. coli,* and H_2S-producing bacteria, lower bacterial population were observed; however, these differences were not found to be statistically significant ($P > 0.05$).

Regarding TVC, the initial bacterial count for control hake fillets was 4.40 log CFU g^{-1}, while the TVC for hake fillets immediately after treatment with CS, CS NP or CASB, was 4.15, 4.15 and 4.04 log CFU g^{-1}, respectively (Fig. 5 a.). However, the initial microbial count found in this study was relatively high compared to the initial TVC of untreated hake fillets caught off the Galician Atlantic coast (North-Western Spain) which was 2.46 log CFU g^{-1}(Garcia-Soto et al., 2013). The higher initial TVC of hake fillets observed may be due to poor handling practises post-mortem such as; manual processing, handling, storage conditions, the skinning process, the time taken to reach the fishery plant, and the hygiene condition of the fishery/processing plant during harvesting (Carrion-Granda et al., 2018; Günlü & Koyun, 2013). Untreated control hake fillets reached the set limit of acceptability on day 6; however, hake fillets treated with either CS, CS NP or CASB did not reach the acceptability limit until after 8, 8.5 and 9 days of storage, respectively. This was a significant ($P < 0.05$) extension of the shelf-life of CS, CS NP, and CASB treated hake fillets compared to untreated control fillets of up to 41, 50 and 55 %, respectively. The longest shelf life was obtained on hake samples treated with CASB; however, this shelf life

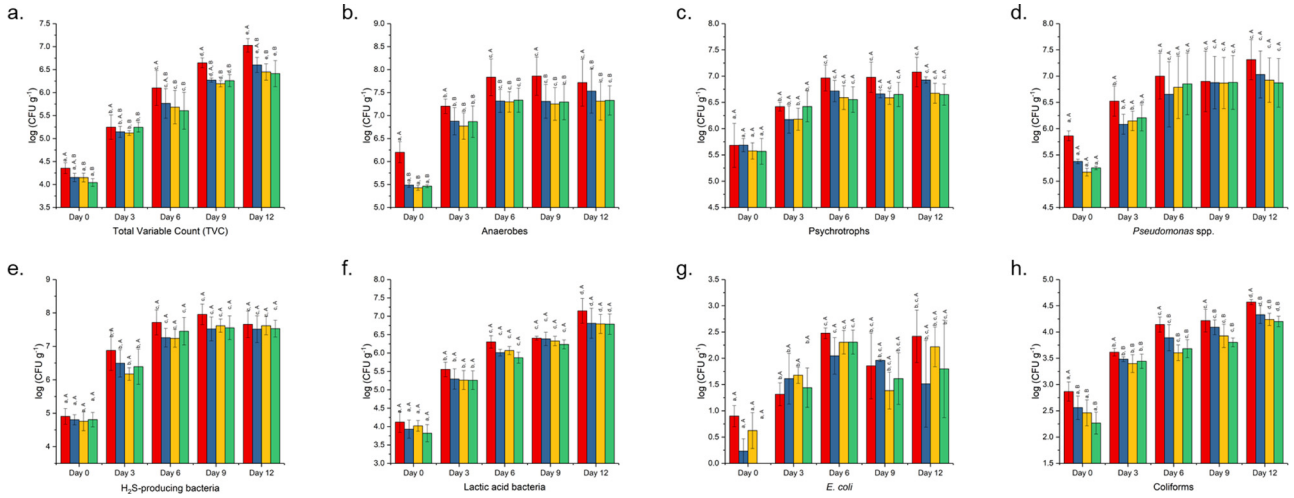

Fig. 5. Microbiological changes of (a) TVC, (b.) anaerobic bacteria (c) psychrotrophic bacteria, (d) Pseudomonas spp., (e.) H_2S-producing bacteria, (f.) LAB, (g.) E. coli, and (h.) total coliform for control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4 °C under vacuum skin packaging over the 12 day storage period. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

extension between antimicrobial treatments was not significantly different. It has been widely reported that CS and CS NPs are more effective antimicrobials materials than essential oil based antimicrobials (Wang et al., 2011); however, the increased shelf life observed in hake fillets treated with CASB is perhaps due to the higher concentration of CASB needed compared with fillets treated with CS or CS NPs to get a similar antimicrobial response. Nevertheless, possible application of a higher concentration of CS or CS NPs solutions for aerosolisation could be used to increase antimicrobial activity; however, a concentration increases of CASB are limited as higher concentrations than 0.8 % are difficult to aerosolise due to an increased viscosity of the solution.

With respect to anaerobic microbial population, a statistical ($P < 0.05$) difference between untreated control and CS, CS NP or CASB treated hake fillets was observed (Fig. 5 b.). The initial anaerobic bacterial count was 6.20, 5.49, 5.43, and 5.46 log CFU g^{-1} for untreated control, CS, CS NP, and CASB treated hake fillets, respectively. The initial microbial count of anaerobic bacteria observed here was high, as other authors such as, (Perez-Alonso et al., 2004), who packaged and stored Atlantic pomfret (*Brama brama*) using VSP at 4 °C found that the initial anaerobe colonies was 4.11 log CFU g^{-1}. The high initial loadings may perhaps be due afore mentioned handling conditions. Nonetheless, a significant ($P < 0.05$) reduction in the anaerobic population of CS, CS NP or CASB treated hake fillets were observed compared to untreated control hake fillet samples.

Regarding psychrotrophic bacteria, results indicate that untreated control hake fillets had greater spoilage compared to CS, CS NP and CASB treated hake fillets; however, this difference was not statistically significant ($P > 0.05$) (Fig. 5 c.). In addition, it was reported that the rate of growth of psychrotrophic bacteria over the storage period was higher compared to TVC and may perhaps be due to the chilled storage of hake fillets at 4 °C preferentially encouraging the growth of psychrotrophic bacteria over mesophilic bacteria (TVC) (Azlin-Hasim et al., 2015).

Similarly, results from *Pseudomonas* spp. bacteria, indicate that untreated control hake fillets had higher microbial spoilage compared to CS, CS NP and CASB treated hake fillets; however, this was not a statistically significant ($P > 0.05$) difference (Fig. 5 d.). From a microbiological perspective the upper limit of 7 log CFU g^{-1} for *Pseudomonas* spp. (Shahbazi et al., 2018) bacteria was not surpassed until day 6 for untreated control hake fillets and not until day 12 for CS treatments. Neither CS NP nor CASB treated hake fillets surpassed the upper limit perhaps due to the antimicrobial effect of the treatments.

Regarding H_2S-producing bacteria, results indicate that untreated control hake fillets had higher microbial spoilage compared to CS, CS NP and CASB treated hake fillets; however, this difference was not statistically significant ($P > 0.05$) (Fig. 5 e.) From a microbiological perspective, the upper acceptable limit for the H_2S producing bacteria of fish products is 6 log CFU g^{-1} (Carrion-Granda et al., 2018) and the upper level of microbial acceptability was surpassed after day 2 of storage for untreated control hake fillets and on day 3 for CS, CS NP or CASB treated hake fillets (Carrion-Granda et al., 2018). These results are in contrast to (Carrion-Granda et al., 2018), who used whey protein isolate coatings incorporated with EOs combined with MAP on hake fillets and found that H_2S-producing bacteria were negligible on day 0. In addition, it has been reported that once *S. putrefaciens* reaches its upper acceptability limit, the bacteria begin to produce sulphur compounds resulting in the spoilage of fish products and an increase in negative organoleptic properties. The high initial microbial count of H_2S-producing bacteria may perhaps be due to afore mentioned handling condition and H_2S-producing bacteria favouring low oxygen environments such as MAP and VSP due to their ability to anaerobically respire (Fernandez-Saiz et al., 2013).

With respect to LAB bacteria, no significant ($P > 0.05$) difference between treatments was observed; however, the microbial load remained below the upper acceptability limit of 9 log CFU g^{-1} (O'Neill, Cruz-Romero, Duffy, & Kerry, 2018) throughout the observed storage period (Fig. 5 f.). However, it has been reported that LAB are not a major spoilage microorganism for fish due to the low storage temperature and the development of an antagonist microflora (Galli, Franzetti, Carelli, Piergiovanni, & Fava, 1993) and our results are in agreement with this. Moreover, the results herein, indicate that Gram-negative bacteria were apparently more susceptible than Gram-positive bacteria, and these results are in agreement with (Azlin-Hasim et al., 2015) and (Galli et al., 1993). The reduced effect of CS, CS NP or CASB treated hake fillets with respect to LAB bacteria, may perhaps be due to LAB's greater resistance to hurdles such as high carbon dioxide levels (found in MAP) (Carrion-Granda et al., 2018) and vacuum-packaging (Fernandez-Saiz et al., 2013). Furthermore, Gram-positive bacteria possess a thicker peptidoglycan layer than Gram-negative, therefore, making it more difficult for antimicrobial materials to penetrate into the cells wall (Azlin-Hasim et al., 2015).

Regarding *E. coli*, no significant ($P > 0.05$) difference between treatments was observed; however, the microbial load of *E. coli* bacteria remained under the acceptability limit of 2.69 log CFU g^{-1} throughout

the observed storage period (O'Neill et al., 2018). Initially counts for *E. coli* bacteria were low to non-existent and the low initial loadings are in agreement with (Fernandez-Saiz et al., 2013) (Fig. 5 g.). However, over the storage period microbial counts of *E. coli* bacteria increased significantly ($P < 0.05$) until day 6 after which they remained relatively constant. While *E. coli* is a facultative anaerobe, it did not surpass the upper acceptability limit during this period; nonetheless, *E. coli* is a serious food spoilage microorganism and its excessive presence in fish can also be indicative of contamination from the local environment (Fernandez-Saiz et al., 2013).

Regarding coliforms, significant ($P < 0.05$) differences between control and antimicrobial treated fillets was observed; however, there was no significant difference between antimicrobial treatments (Fig. 5 h.). Independent of antimicrobial treatment, a significant increase in the microbial population of coliform bacteria until day 12 was observed; nevertheless, the microbial load did not surpass the upper acceptability limits of 5 log CFU g^{-1} (García-Soto, Fernández-No, Barros-Velázquez, & Aubourg, 2014). Moreover, low initial counts of coliform bacteria have been reported elsewhere by (Volpe et al.) who coated rainbow trout fillets with a carrageenan enriched with essential lemon oil. These result indicate good hygiene conditions in the fishery plant (Shahbazi & Shavisi, 2018).

In general, when comparing the shelf life of CS, CS NP, and CASB treated hake fillets; nanomaterials treatments of CS NPs and CASB gave a slightly longer shelf-life than non-nano CS; however, this was not statistically significant ($P > 0.05$). This may be due to the fact that the antimicrobial activity of for CS and CS NP materials have been reported to be similar due to the strong solvent swelling character and CS:TPP composition of CS NPs affecting the amount of free amine and therefore, the antimicrobial activity of CS NPs (Ristić, Lasič, Kosalec, Bračič, & Fras-Zemljič, 2015; Sullivan, Cruz-Romero et al., 2018). Moreover, CS and CS NP have a similar modes of antimicrobial action which is not fully understood; however, several modes of action have been proposed such as; (i) positively charged CS interacting with the negatively charged components of the bacterial cell walls, resulting in cytoplasm leakage from the microorganism, (ii) through its ability to form a layer surrounding the bacterial cell, therefore, inhibiting the absorption of nutrients, and resulting in cell death (Sullivan, Azlin-Hasim et al., 2018), or (iii) low molecular weight CS can bind to DNA and inhibit RNA replication due to its ability to penetrate towards the nuclei of microorganisms. Furthermore, the Gram-strain may also affect the mode of antimicrobial action as high molecular weight CS can form a film on Gram-positive bacteria preventing uptake of nutrients while low molecular weight CS could interfere with cellular metabolism on Gram-negative bacteria due penetration of CS into the cell (Sullivan, Cruz-Romero et al., 2018). Regarding the marginally longer shelf life seen by CS NPs, may perhaps be associated with NPs increased surface area compared to their bulk counterpart thus allowing more interaction with the bacterial cell components (Ngan et al., 2014) and in addition CS NPs greater monodispersity may allow for more efficient aerosolisation and, therefore, a greater antimicrobial coating on the hake fillet surface. Regarding CASB, the mode of antimicrobial activity differs from CS and CS NP and likewise, the exact antimicrobial mechanism of action of EO's based materials is not fully understood; however, it is believed to be from the synergistic action of constituent secondary metabolites commonly found in EO's. Carsonic acid is phenol diterpenoid that has been associated with the antimicrobial activity of rosemary essential oil (Abdollahi et al., 2014) and is a compound that can affect the cellular membrane allowing for the passive transport of ions through the membrane and can disintegrate outer membranes of Gram-negative bacteria or in Gram-positive bacteria alter he membrane permeability, allowing permeation cations like H^+ and K^+ (Sullivan, Azlin-Hasim et al., 2018). Moreover their greater antimicrobial activity may perhaps be due to enhanced antimicrobial activity of nano-solubilisates (Cruz-Romero, Murphy, Morris, Cummins, & Kerry, 2013) and afore mentioned concentration difference.

Overall, the results confirmed that the application of natural antimicrobials can enhance the shelf life of fish products but that the initial microbial load will affect significantly the shelf life of these products; therefore, to enhance safety and shelf life of fish products through the application of any natural antimicrobial should start with the highest quality product as shelf life enhancement will be defined by the initial bacterial count and as for any preservation technology, the initial quality of fish products cannot be enhanced.

4. Conclusions

Results obtained herein indicate that the microbiological and physiochemical properties of hake fillets aerosolised with CS, CS NP or CASB antimicrobial coating solutions as part of a hurdle strategy significantly ($P < 0.05$) enhanced shelf life in comparison to control treated hake fillets. The best shelf life was observed using CASB treatments which increased the shelf life by up to 50 % upon control treatments. Therefore, aerosolisation treatment of hake fillets with NAM antimicrobial solutions was an effective hurdle in VSP packed hake fillets. This research highlights the potential of aerosolisation treatments which require small volumes of antimicrobial solutions, give good coating coverage, and have a minimal impact on physiochemical parameters such as proximal analysis. Furthermore, another potential advantage of the aerosolisation treatments of food products could be the minimal effect this type of treatment will have on the organoleptic properties of the treated samples since the coating is a thin layer which makes us believe that organoleptic properties of the hake fillets will not be negatively impacted; but further studies are required. Moreover, aerosolisation treatment could be a suitable method to treat a diverse range of high value, delicate or perishable food products including; fruits, vegetables, chicken, beef and seafood. Additionally, the shelf extension from aerosolisation treatments could be further improved upon through the manipulation of aerosolisation parameters such as flow rate, antimicrobial solution concentration and exposure times and use of other antimicrobial nanomaterial solutions such as EO nanoemulsions. Overall the results of this study demonstrate that the aerosolisation treatments of nano and non-nano natural antimicrobial materials are an effective hurdle to extend the shelf-life of hake fillets.

CRediT authorship contribution statement

David J. Sullivan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Malco C. Cruz-Romero:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Project administration. **Ana B. Hernandez:** Investigation, Formal analysis. **Enda Cummins:** Funding acquisition. **Joseph P. Kerry:** Writing - review & editing, Funding acquisition, Supervision. **Michael A. Morris:** Writing - review & editing, Funding acquisition, Supervision.

Acknowledgements

This work was funded under the National Development, through the Food Institutional Research Measure (FIRM) administered by the Department of Agriculture, Food and the Marine, Republic of Ireland.

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