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Chitosan films for the microbiological preservation of refrigerated sole and hake fillets

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ABSTRACT

The effect of chitosan films for shelf-life extension of fresh fillets of hake (*Merluccius merluccius*) and sole (*Solea solea*) was evaluated over a 15-day storage at 4 ± 1 °C. Fish fillets wrapped in a chitosan matrix were individually packaged in air (AP) and under vacuum (VP). Microbiological analyses (total aerobic mesophilic bacteria, *Pseudomonas*, H_2S-producing bacteria, lactic acid bacteria, Enterobacteriaceae and *Listeria monocytogenes* were carried out during the shelf-life. The microbial species were inhibited ($p < 0.05$) by the presence of chitosan films, especially under VP conditions. A significant ($p < 0.05$) increase of the lag phase and a reduction of the final microbial population were detected mainly for total aerobic mesophilic bacteria, H_2S-producing bacteria and *Pseudomonas*. The present work demonstrates the biocide effectiveness of chitosan as an internal layer of the packaging material, offering a promising alternative which would enhance the quality of fish during refrigerated storage.

1. Introduction

Fish products are certainly the most difficult fresh foodstuffs to preserve because of their high water contents (65–80%), *post mortem* pH 6–7 and large amounts of non-protein nitrogen (9–18% N_{tot}) (Franzetti, Martinoli, Piergiovanni, & Galli, 2001). Consequently, marine-based products are highly perishable during refrigerated storage, mainly due to rapid microbial growth of microbiota or from contamination. This can result in quality or safety issues and therefore associated with either economic or health-related problems.

Besides traditional methods used to extend the shelf-life of fish and fishery products, such as rapid chilling and ice storage, various strategies involving modified atmosphere packaging, ionizing radiation and the use of organic acids or other antimicrobial agents have been proposed to inhibit spoilage and pathogenic microorganisms (Arkoudelos, Stamatis, & Samaras, 2007; Chouliara, Savvaidis, Panagiotakis, & Kontominas, 2004; Mejholm & Dalgaard, 2002; Pantazi, Papavergou, Pournis, Kontominas, & Savvaidis, 2008).

Since society is experiencing a trend towards 'green consumerism', with a desire of fewer synthetic food additives and products with a smaller impact on the environment, the use of naturally derived antimicrobials has substantially increased in the last decade. In this sense, chitosan (β-(1.4)-2-amino-2-deoxy-D-glucose) has attracted much attention. Chitosan has excellent film forming properties, and derives from chitin, the second most abundant natural polymer in nature after cellulose. It is mostly available from waste bio-products in the shellfish industry, and therefore, abundant commercial suppliers are offered currently. It can also be obtained from the chitin component of fungal cell walls.

Several studies have already demonstrated the antibacterial and antifungal action of this compound for both bioactive preservative and bioactive packaging applications (Fernandes et al., 2008; Li et al., 2007; No, Meyers, Prinyawiwatkul, & Xu, 2007; No, Park, Lee, & Meyers, 2002; Qin et al., 2006; Rhoades & Roller, 2000). Concerning the mode of action of chitosan films, the biocide effect has already been well demonstrated that is generally related to the release from the biopolymer of protonated glucosamine fractions into the culture medium (Fernandez-Saiz, Lagaron, Hernandez-Muñoz, Ocio, 2008; Fernandez-Saiz, Ocio, & Lagarón, 2006). Since the presence of water is an indispensable factor for the glucosamine chains release and, therefore, for the antimicrobial performance of chitosan-based films, fish could be a very suitable target food product due to its high water content.

The antimicrobial activity of chitosan in fish products is well established in the literature, and chitosan-based coatings have already been used for a variety of fish species to reduce microbes

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and hence improve overall fish quality and extent its shelf-life (reviewed by Alishahi & Aider, 2012). For instance, Jeon, Janak & Shahidi (2002) evaluated samples of chitosan-coated Atlantic cod and herring and showed a considerable reduction of total viable counts (TVC). Tsai Su, Chen, & Pan (2002) studied the effect of chitosan solutions on salmon fillets and obtained an extension of the shelf-life from 5 to 9 days. Moreover, Lopez-Caballero, Gomez-Guillen, Perez-Mateos, and Montero. (2005) demonstrated that a chitosan-gelatin coating prevents spoilage of cod patties due to a decrease in total volatile basic nitrogen and in the microorganism counts, in particular counts of Gram-negative bacterial counts. Duan, Jiang, Cherian, and Zhao (2010) reported that the combination of a chitosan coating and vacuum packaging results in ~2-log reductions in total plate counts after 14-days storage of fresh lingcod. Vasconez, Flores, Campos, Alvarado, and Gerschenson (2009) demonstrated that chitosan coating extends the quality of sliced salmon to six days. All the above-mentioned studies show that chitosan coatings have applied successfully on fish products to extent shelf-life however, application of chitosan films is scarce Fernandez-Saiz, Soler, Lagaron, and Ocio (2010) have recently demonstrated inhibitory and bactericidal effects when chitosan films were introduced into fish soup samples in which *Staphylococcus aureus*, *Salmonella* spp. and *Listeria monocytogenes* were previously inoculated. Ye, Neetoo, and Chen (2008) reported that chitosan films did not have any effect on *L. monocytogenes* inoculated on cold-smoked salmon. Vacuum-packaged chitosan films prolonged the shelf-life of sea bass fillets by about 20 days (Günlü & Koyun, 2012).

In Spain and certainly in other European countries, gadoid fish represent an important percentage of overall fish consumption. Within this group, hake is in great demand by consumers. The capture of European hake in distant fishing banks usually means that the time elapsed between the catch and the arrival at its destination may vary from 3 to 10 days, underlining the need to optimize refrigeration parameters to provide consumers with fish of the highest quality possible (Rodríguez, Losada, Aubourg, & Barros-Velázquez, 2004). Similarly, sole and senegalese sole have been firm candidates for marine culture in Europe for decades. Technological limitations have been the primary restriction for commercial implementation of these species at present (Tejada, De las Heras, & Kent, 2007).

In the present study, we assessed the possibility of improving the shelf-life of fresh sole and hake fillets by using low-molecular-weight chitosonium acetate films in air and vacuum-packaged during 4 °C storage.

2. Materials and methods

2.1. Preparation of antimicrobial films

Chitosan polysaccharide with low molecular weight (83.3% degree of deacetylation and viscosity of 115 mPa·s at 1% in 1% acetic acid as stated by the manufacturer) was purchased from Sigma–Aldrich (Madrid, Spain). Chitosan dispersions were prepared in 2% (v/v) acetic acid to a final concentration of 3% (w/v) and stirred at 37 °C for approx. 3 h. Chitosonium acetate solution was filtered through polyester cloth to remove residues of insoluble particles and then autoclaved before film formation. Neat chitosan-based films of ca. 25 microns of thickness were obtained by casting onto 90 mm polystyrene (PS) Petri dishes and subsequent solvent evaporation at 37 °C for 18 h. Their effectiveness was tested immediately after film formation in order to avoid chemical changes during storage which could have an effect on the antimicrobial properties (Fernandez-Saiz et al., 2010; No, Kim, Lee, Park, & Prinyawiwatkul, 2006). Previous trials performed by the authors demonstrated that the incorporation of chitosonium acetate films into the culture medium led to pH reduction levels (from 6.2 to 5.7) which did not show biocide properties (Fernandez-Saiz et al., 2006, 2010). In the current work, the pH values of hake and sole after the packaging system with chitosonium acetate films were 5.8 ± 0.2 and 6.7 ± 0.2, respectively.

2.2. Bacterial strain and preparation of the inoculum

L. monocytogenes CECT 5672 was obtained from the Spanish Type Culture Collection (Valencia, Spain). The strain was stored in Tryptone Soy Broth (TSB; Conda Laboratories, Pronadisa, Madrid, Spain) with 20% glycerol at −80 °C until needed. For experimental use, the stock culture was maintained by regular subculture to agar Tryptone Soy Agar (TSA; Conda Laboratories) slants at 4 °C and transferred monthly. A loopful of bacteria was removed to 10 mL of TSB and incubated at 37 °C overnight. A 100 μL aliquot from the overnight culture was again transferred to TSB and grown at 37 °C to the mid-exponential phase of growth. The CFU counts were accurately and reproducibly obtained by inoculation of 0.1 mL of a 100-fold diluted culture having an absorbance value of 0.15 as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy using an SP-2000 UV spectrometer (Spectrum Instruments Company, Shanghai, China).

2.3. Challenge tests

Locally purchased fresh fillets of hake (*Merluccius merluccius*) and sole (*Solea solea*) were used in this study. *L. monocytogenes* was independently inoculated on 25 g of fish fillet as to achieve concentrations of about $2–4 \times 10^3$ CFU/g. The effectiveness of the antimicrobial chitosan films was evaluated by placing the films (4×7 cm^2) on the upper and the bottom surfaces of the fish slices. Thereafter, fish samples were individually packed in high oxygen barrier multilayers obtained by co-extrusion process (PE/EVOH-PA-ION/PE; thickness: 80/10/10 μm) and vacuum thermosealed using a vacuum chamber machine (Multivac A300/52 Vacuum, Packaging Systems, S. L., Madrid, Spain). The EVOH based high barrier layer was a blend containing 5 wt% of an amorphous polyamide (PA) and an ionomer (ION). Fish samples were stored at two packaging conditions, air packaging (AP) and vacuum packaging (VP) under refrigeration (4 ± 0.5 °C) for 15 days. To monitor the antimicrobial activity of the films, duplicate samples from each condition were removed at different interval times (days 0, 1, 3, 6, 8, 6, 10 and 15) for microbial enumeration. Control samples were stored at the above conditions without the presence of the chitosan films.

2.4. Microbiological analyses

Each sample was aseptically unwrapped from its packaging and transferred to a bag (Bag Page, Interscience, St-Nom-la-Bretèche, France) Subsequently, 225 mL of peptone water (peptone 10%, NaCl 5%, purified water 85%, final pH: 7.4) were added and the mixture was homogenized for 120 s in a Pulsifier® (Microgen Bioproducts Ltd., Surrey, UK). Samples (0.1 mL) of serial dilutions of fish homogenates were spread on the surface of the dry media in Petri dishes and incubated as follows.

Aerobic mesophilic counts were determined in plate count agar (PCA; Conda Laboratories) at 30 °C for 72 h.

Enterobacteriaceae counts were enumerated on violet red bile glucose agar (VRBGA, Conda Laboratories) incubated at 37 °C for 48 h. Only large colonies with purple haloes were counted.

The H_2S-producing bacteria (typical of *Shewanella putrefaciens*) counts were determined in iron agar (IA, Conda Laboratories) supplemented with 4% w/v sterile filtered L-cysteine (Gram, Trolle,