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Survival of *Listeria monocytogenes* and *Salmonella* Enteritidis in sea bream (*Sparus aurata*) fillets packaged under enriched CO_2 modified atmospheres

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ABSTRACT

The effect of packaging of fresh sea bream fillets under different modified atmospheres (high levels of CO_2 combined with N_2) on the behaviour of *Listeria monocytogenes* and *Salmonella* Enteritidis was studied. Two different strains of each pathogen were spread over the sea bream fillets stored at either 0 °C or 4 °C for 16 days under standard supermarket lighting conditions (14 h a day). The atmospheres investigated were 60% CO_2/40% N_2, 70% CO_2/30% N_2, 80% CO_2/20% N_2 and air as control. Mesophilic, psychrotrophic and *Enterobacteriaceae* microbial counts were also performed in control batches. The results indicate that all the CO_2 concentrations used favoured the control of pathogen bacteria inoculated, when compared with air conditions especially, when combined with storage at 0 °C. Both storage temperature (0 °C and 4 °C) resulted in a decrease in *Salmonella* count. The results were more significant for *Listeria* strains, because during the storage at 4 °C *Listeria* count increased in all batches, while for samples stored at 0 °C a significant reduction in microbial load except in control batch was observed. Sensitivity differences of inoculated strains against temperature and CO_2 concentrations were also discussed.

1. Introduction

Total worldwide aquaculture production of sea bream in 2010 was 139,925 t according to FEAP (The Federation of European Aquaculture Producers) (Apromar, 2011). Sea bream is one of the most cultivated species in the Mediterranean countries being the main producers: Greece, Turkey and Spain covering 81.1% of the world production in 2010 (Apromar, 2011). Nowadays consumers demand safe fresh products without additives with long shelf life preparations. These requirements entail the necessity to improve packaging and preservation methods for fresh fish. One of the most important and efficient technologies used is Modified Atmosphere Packaging (MAP) combined with refrigeration. MAP is a hyperbaric process that includes removal or replacing the atmosphere, using a specific combination of various gases, such as carbon dioxide, nitrogen and oxygen (Nettles Cutter, 2002). Although this atmosphere undergoes alterations over time, it cannot be reset during storage (Jay et al., 2009). The effectiveness of MAP has been known since the late 20s, but is not used commercially as a method of packaging in Europe until the 70s (Phillips, 1996). The appropriate combination of gases depends on the product, the packaging material, the storage temperature and other factors, such as the initial microbiological quality or the presentation of the product (Mejía and García, 2007). In many studies it was decided to remove oxygen from the gas mixture for packaging some fish species (Arashisar et al., 2004; Goulas and Kontominas, 2007; Özogul et al., 2004; Pantazi et al., 2008; Stamatis and Arkoudelos, 2007a,b) trying to reduce aerobic microbial growth, some enzymatic reactions and lipid oxidation (Ray and Bhunia, 2008). On the other hand, the lack of growth of some spoilage microorganisms could allow some pathogens such as *Clostridium botulinum* and *Listeria monocytogenes* to grow (Palumbo, 1988). Hence, packaged products must be kept at refrigeration temperatures, below 3.3 °C, if possible, in order to extend their shelf-life (Forsythe, 2003). Carbon dioxide is commonly used due to its antimicrobial effect on reducing the lag phase and generation time of microorganisms and this effect is intensified by the use of low temperatures. In addition, there are different mechanisms that enhance this antimicrobial effect, such as the lowering of the pH and the interference with certain enzymes like succinic oxidase, regarding the decarboxylation or the dehydration of the bacterial cell membrane leading to prevention of the transport of soluble solutes into the cell (ICMSF, 1980). Nitrogen, is normally used as filler gas in order to prevent package collapse (Coles et al., 2003).

Concerns have been expressed about the fact that the increase in shelf-life of MAP products may provide sufficient time for human pathogens to multiply to levels which render the food unsafe before the end of its shelf life (Jay, 1992). The conditions used in MAP technology that avoid the suppression of indigenous flora in combination with refrigeration temperatures and the intrinsic characteristics of fresh fish may enhance the growth of some pathogens. *Listeria* is a gram-positive bacterium, psychrotrophic, facultative anaerobic, widely distributed in nature and is able to survive for long periods in food,

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constituting a major risk for fresh fish in modified atmosphere packaging (Ray and Bhunia, 2008). The most common infection is by ingestion of ready to eat products as marinated or cold smoked fish (FAO/WHO, 2004) which are not subjected to any heat treatment before consumption but the risk of Listeriosis could also increase in some geographic areas where fresh fish can be consumed raw or undercooked (Feldhusen, 2000), especially among risk groups, such as children, elderly, immunosuppressed people or pregnant women (AESAN, 2009). Processing is the most crucial step as far as contamination of the product is concerned (Soultos et al., 2007). *Salmonella* is one of the most important pathogens associated with gastrointestinal diseases. *Salmonella* is a gram negative, facultative anaerobic mesophilic microorganism, member of *Enterobacteriaceae* family and is also widely distributed in nature, with human and animal gastrointestinal being the main vehicle of this pathogen. Despite the low incidence in fresh fish (AESAN, 2009) and that these bacteria are not psychrotrophic and can be easily inactivated by heat, the nature of the product of study that sometimes can be eaten undercooked means that there is also some risk of infection. There are also risks associated with food processing as it can cause cross-contamination with other products while being manipulated (Nickelson et al., 2001).

The objective of this study was to determine the effect of modified atmospheres enriched with CO_2 at refrigeration temperatures (0 °C ± 1 °C and 4 °C ± 1 °C) on the behaviour of *L. monocytogenes* and *S.* Enteritidis during storage of fresh gilthead sea bream fillets. The effectiveness of the treatments depending on the inoculated strain was also studied.

2. Materials and methods

2.1. Preparation of fish samples

Three hundred aquacultured gilthead sea bream (*Sparus aurata*) were supplied by Doramar (Aquicosta S.L) from the FAO catch area number 37 on the Spanish coast. Fishes were received at the laboratory 18–24 h after capture in expanded polystyrene boxes with flake ice and were gutted and filleted by hand, washed with cold running water and packaged individually in trays and divided into four batches; three MAP samples 60% CO_2/40% N_2 (60/40), 70% CO_2/30% N_2 (70/30), 80% CO_2/20% N_2 (80/20), and air (control batch). The packaging of MAP samples was carried out in polystyrene trays sealed with a polyethylene/polyamide laminate film with a water vapour permeability of 5–7 g/m^2/day at 23 °C and an oxygen permeability of 40–50 cm^3 mm/m^2 day atm at 23 °C (Irma, Zaragoza, Spain) in a packaging machine (ULMA SMART-400, Spain). The average weight was 423.0 ± 66.0 g and 157.7 ± 10.1 g for whole and filleted fishes respectively with an initial pH of 5.7 ± 0.1. The packaged fillets were kept refrigerated during the inoculation of all samples (less than 1 h) as well as control batch (non-inoculated).

2.2. Preparation of inocula

The selected freeze-dried cultures were reconstituted according to the recommendations of the CECT (Spanish Type Culture Collection). Two strains of *L. monocytogenes* (CECT 935 and CECT 7467) and two strains of *S.* Enteritidis (CECT 4155 and CECT 4300) were selected. Lyophilized strains were reconstituted in brain heart infusion broth (BHI broth, Oxoid, Basingstoke, Hampshire, England) during 24 h at 37 °C. After verifying the purity of cultures, they were incubated in trypticase soya agar (TSA, Oxoid) tubes and kept refrigerated until the time of the inocula preparation. The preparation of the suspensions was conducted in three phases. Bacterial cells were transferred from TSA tubes to TSA plates and the plates were incubated at 37 °C for 24 h in order to isolate clear colonies. The isolated colonies were transferred in 10 ml tubes of BHI and were incubated with stirring for 6 h at 37 °C (to achieve exponential growth phase). The portions (1 ml) were transferred after the incubation for 6 h in flasks with 50 ml of BHI broth and the flask was incubated with stirring for 24 h at 37 °C (to achieve growth in stationary phase). Finally portions (1 ml) of the 24 h culture were transferred in 50 ml of sterile peptone water (Biolife, Milan, Italy), for each strain of *Listeria* and *Salmonella*.

2.3. Inoculation of samples and preparation of the batches

1 ml of the inoculated peptone water was spread over the dorsal–cranial half surface of the fillets with sterile 1-ml syringe and 0.6 mm × 25 mm needle (BD Plastipak, Madrid, Spain). This part of the fillet was always used to collect the analytical samples. The count after inoculation evidenced that the inoculum size injected was almost fully recovered.

Finally, 20 batches were obtained by combining the four atmospheres used (60/40, 70/30, 80/20 and air) with the five treatments (the *L. monocytogenes* 935, *L. monocytogenes* 7467; *S.* Enteritidis 4155, *S.* Enteritidis 4300 inoculated samples and the control batch without inoculum). Each batch was composed by fifteen trays with a single fillet inside each package. Three trays (three fillets from three different fishes) were analyzed each sampling day from each batch. Sampling was carried out at predetermined time intervals (0, 4, 8, 12, 16 days) starting with an initial point just after inoculation. The experiment was carried out at 0 °C and 4 °C in standard supermarket lighting conditions (14 h a day) during 16 days of storage.

2.4. Microbiological analyses

For uninoculated samples (control) the total mesophilic and psychrotrophic viable count (MVC and PVC), *Enterobacteriaceae*, *Salmonella* investigation (presence/absence) were studied during storage at 0 and 4 °C. For MVP, PVC and *Enterobacteriaceae* analyses, 10 g of fish muscle was taken from the dorsal region of each fillet, transferred aseptically into a stomacher bag (Seward Medical, UK), mixed with 90 ml of 0.1% peptone water containing 1% NaCl and homogenised for 60 s using a Stomacher (Lab Blender 400, Barcelona, Spain). MVC and PVC were determined by pour plate methods in Plate Count Agar (Merck, Darmstadt, Germany) using conventional dilution procedures. Plates were incubated 48 h at 37 °C for MVC and 7 days at 10 °C for PVC. For *Enterobacteriaceae*, violet red bile dextrose agar (VRBD, Scharlab, Barcelona, Spain) with double layer was used and plates were incubated 48 h at 37 °C.

25 g of these fillets was taken to determine the presence or absence of *Salmonella* as described by Allaert-Vandevenne and Escolá-Ribes (2002). The investigation of *Listeria* was carried out following the UNE-EN/ISO 11290-2 detection method with modifications according to UNE-EN/ISO 11290-2:2000/A1.

For *Listeria* inoculated samples, decimal dilutions were made from 10 g muscle and 90 ml of buffered peptone water. Portions (0.01 ml) were taken from the appropriate decimal dilutions and surfaced platted in duplicate on *Listeria* chromogenic agar (Oxoid) and count were performed after incubating the plates during 24–48 h at 37 °C. The procedure for *Salmonella* inoculated samples was performed similarly (Allaert-Vandevenne and Escolá-Ribes, 2002). Appropriate dilutions were surface platted on Xylose, Lysine, Deoxycholate Modified agar (Scharlab) and incubated 24–48 h at 37 °C. Biochemical tests were performed to confirm *Salmonella* and adjust the obtained count by plating suspicious XLD colonies on Bismuth Sulfite Agar and Brilliant Green Agar (Panreac, Barcelona, Spain). These colonies were also punctured into Triple Sugar Iron Agar and Lysine Iron Agar tubes (Panreac) and the urease test was also performed.

2.5. Gas analyses

Headspace gas composition of control batches was measured throughout the display time with a gas analyzer (Witt-Gasetechnic,