International Journal of Food Microbiology 166 (2013) 141–147

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Survival of *Vibrio parahaemolyticus* and *Aeromonas hydrophila* in sea bream (*Sparus aurata*) fillets packaged under enriched CO_2 modified atmospheres

Laura Provincial, Elena Guillén, Verónica Alonso, Mario Gil, Pedro Roncalés, José A. Beltrán *

Department of Animal Production and Food Science, Laboratory of Science and Technology of Meat and Fish, Faculty of Veterinary Sciences, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain

ARTICLE INFO

Article history:
Received 18 March 2013
Received in revised form 6 June 2013
Accepted 8 June 2013
Available online 28 June 2013

Keywords:
Modified atmosphere packaging
Sea bream
Vibrio
Aeromonas
CO_2

ABSTRACT

The ability to survive of two pathogens (*Vibrio parahaemolyticus* and *Aeromonas hydrophila*) spread over sea bream fillets packaged under different modified atmospheres (MAPs) was studied at 0 °C and 4 °C under refrigerated storage. The atmospheres used were 60% CO_2/40% N_2, 70% CO_2/30% N_2 and 80% CO_2/20% N_2 and a control batch packaged in air. Head space gas analyses, microbial counts and confirming test of pathogenic bacteria were carried out during 16 days. The results obtained showed that all the modified atmospheres studied were effective to reduce the microbial load of sea bream fillets when compared with air packaged samples although small differences were found among MAPs. Temperature storage was the main factor to reduce microbial growth. *V. parahaemolyticus* was unable to grow at both temperatures, 0 °C and 4 °C (except air batches) while *A. hydrophila* showed significant growth at 4 °C and microbial inactivation at 0 °C.

1. Introduction

Aquaculture is the fastest growing sector of the world food economy and currently accounts for more than 45.7% of the world's fish production for human consumption in 2008 (FAO, 2010). One of the most important farmed fish species in Mediterranean countries is sea bream (*Sparus aurata*) being Greece, Turkey and Spain as the main producers covering 81.1% of the world production in 2010 (Apromar, 2011). The consumer requirements of fresh, safe, attractive, easy-buy, easy-consume and longer shelf-life products entail the necessity to improve the packaging and preservation methods for fresh fish. MAP (modified atmosphere packaging) combined with refrigeration presents numerous advantages because it extends product shelf-life, reduces economic loss and distribution costs and allows the product to be transported to longer distances for distribution and to be easily exposed for marketing and sealing (Phillips, 1996). On the other hand, one of the major MAP disadvantages is that the increase in shelf-life may provide sufficient time for human pathogens to multiply due to the suppression of indigenous flora in combination with refrigeration temperatures and the intrinsic characteristics of fresh fish (Jay et al., 2009).

Vibrio parahaemolyticus is the most commonly isolated "noncholera" *Vibrio* (Feldhusen, 2000). Since *V. parahaemolyticus* was recognised as a pathogen for humans, the reported cases of disease caused by ingestion of food contaminated with this pathogen around the world have been increasing, especially in Asian countries (Lin et al., 2004) where consumption of fish and fish products is higher and the food culture is characterised by an undercooked consumption. The optimal temperatures for growth of *V. parahaemolyticus* range from 35 °C to 37 °C although minimal temperatures have been reported to be as low as 4 °C or 5 °C (Beuchat, 1982; Johnson et al., 1973). *Vibrio* spp. could also be affected by atmosphere packaging and CO_2 could decrease microbial count as in many Gram-negative bacteria (Arkoudelos et al., 2007; Kimura and Murakami, 1993). The incidence of the disease in Asia, Europe and the United States diverges significantly. In the Asian region, *Vibrio* spp. have been recognised as the leading cause of foodborne outbreaks in many countries including China (Yang et al., 2008), India (Chakraborty et al., 2008) or Malaysia (Noorlis et al., 2011). An estimated 4500 cases of *V. parahaemolyticus* infection occur every year in the United States. However, the number of cases reported to the CDC (Center for Disease Control and Prevention) is much lower because surveillance is complicated by underreporting (CDC, 2009). In 2001, the Scientific Committee on Veterinary Measures Relating to Public Health of the European Commission concluded that *V. parahaemolyticus* outbreaks are rarely reported in Europe (European Commission Health and Protection Directorage-General, 2001) because the risk of *V. parahaemolyticus* infection is extremely low in Europe. Davies et al. (2001) reported 11% of positive samples from fresh fish purchased from commercial outlets in France, Great Britain, Greece and Portugal. However, data obtained from clinical journals and from unreported cases of *V. parahaemolyticus* infections identified at hospitals have shown that *V. parahaemolyticus*

* Corresponding author. Tel.: +34 976762738; fax: +34 976761612.
E-mail address: jbeltran@unizar.es (J.A. Beltrán).

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.06.013

infections in Spain are more common than previously assumed (Martinez-Urtaza et al., 2005).

Over the past twenty five years, *Aeromonas* have received increasing attention as an emergent agent of foodborne gastrointestinal disease (Pablos et al., 2009). The ability of *Aeromonas* to grow at refrigeration temperatures may have great impact on refrigerator-stored foods (Daskalov, 2006). Atmosphere composition could also affect *Aeromonas* growth and there are several reports on the isolation of *Aeromonas* from vacuum packed and modified atmosphere packed foods (Doherty et al., 1996; Hudson et al., 1994; Ravi Sankar et al., 2008). While *Aeromonas* have been isolated from fish, shellfish, meats, dairy products, and fresh vegetables, few foodborne outbreaks have been reported (EPA. United States Environmental Protection Agency, 2006). Worldwide occurrence of *Aeromonas* spp. outbreaks could vary between countries but in Spain, data from the National Epidemiology Centre (Epidemiological Surveillance System, 2007) indicate that *Aeromonas* spp. occupied the fourth place among causes of total gastrointestinal diseases reported each year over the period 1997 to 2006.

Therefore, the consumption of fish and seafood contaminated with these bacteria involves a great risk for public health. Although both pathogens could be easily inactivated by heat treatments during cooking (ICMSF, 1980a; Saxena and Kulshrestha, 1985), the magnitude of the risk becomes clearer when the shift in the eating habits of people in Europe and in many other parts of the world towards raw or undercooked seafood (Broglia and Kapel, 2011) is taken into consideration.

The objectives of this study were to determine the effect of different CO_2/N_2 atmospheres on the survival of *V. parahaemolyticus* and *Aeromonas hydrophila* during storage of fresh gilthead sea bream fillets. Effectiveness of the treatments depending on the inoculated strain was also studied as well as the susceptibility of these strains to refrigeration temperatures (0 °C and 4 °C).

2. Materials and methods

2.1. Preparation of fish samples

Three hundred aquacultured gilthead sea bream (*S. aurata*) from the FAO capture zone 37 (Spanish coast), were provided by Doramar (Aquicosta S.L.). Fishes were received in the laboratory 24 h postharvesting in expanded polystyrene boxes with flake ice. After gutting, filleting and washing by hand, fishes were packaged individually 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 $cm^3 \cdot mm/m^2 \cdot day \cdot atm$ at 23 °C (Irma, Zaragoza, Spain) in a packaging machine (ULMA SMART-400, Spain). Samples were divided into four batches: 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 a control batch packaged in air. The packaged samples were kept refrigerated during the inoculation of all samples (less than one hour) as well as the control batch (noninoculated). The average weights were 411.0 ± 57.0 g and 137.1 ± 16.5 g for whole and filleted fishes respectively with an initial pH of 6.2 ± 0.2.

2.2. Preparation of inocula and inoculation procedure

The selected freeze-dried cultures were reconstituted according to the recommendations of the CECT (Spanish Type Culture Collection). Two strains of *V. parahaemolyticus* (CECT 511 and CECT 5304, named V511 and V5304) and two strains of *A. hydrophila* (CECT 5216 and CECT 5734, named A5216 and A5734) were selected. The *Vibrio* freeze-dried cultures were reconstituted in alkaline peptone water (APW, Merck, Darmstadt, Germany) for 24 h at 37 °C. After verifying the purity of cultures, they were incubated in TSA tubes (Trypticase Soya Agar, Oxoid, Basingstoke, Hampshire, England) and kept refrigerated until the time of the inoculum preparation. The preparation of the suspensions was conducted in three phases. Bacterial cells were transferred from TSA tubes to TSA plates for the isolation of colonies after incubation for 24 h at 37 °C. The isolated colonies were transferred into 10 ml tubes of APW and were incubated with stirring for 6 h at 37 °C (to achieve exponential growth phase). Portions (1 ml) of this media were diluted in flasks with 50 ml of APW and were incubated with stirring for 24 h at 37 °C (to achieve growth in stationary phase). Finally, portions of 1 ml of the 24 h culture were diluted in 50 ml of sterile peptone water (Biolife, Milan, Italy), for each *V. parahaemolyticus* strain. In the case of *A. hydrophila* the reconstitution and subsequent incubation were conducted in BHI (brain heart infusion, Oxoid) instead of APW and the incubation temperature used was 30 °C.

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 microbial 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 (*V. parahaemolyticus* 511, *V. parahaemolyticus* 5304, *A. hydrophila* 5216 and *A. hydrophila* 5734 inoculated samples and the control batch without inoculum). Each batch was composed of fifteen trays with a single fillet inside each package. Three trays (three fillets from three different fishes) were analysed 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 study was carried out at 0 °C ± 1 °C and 4 °C ± 1 °C in standard supermarket lighting conditions (14 h a day) during 16 days of storage.

2.3. Microbiological analyses

Total mesophilic and psychrotrophic viable count (MVC and PVC), Enterobacteriaceae, *Vibrio* investigation (presence/absence) and *Aeromonas* investigation (presence/absence) were carried out in control samples (noninoculated fillets). Ten 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) for MVC, PVC and Enterobacteriaceae count. 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 with double layer was used (VRBD, Scharlab, Barcelona, Spain) and plates were incubated for 48 h at 37 °C.

Twenty-five grams of these fillets was taken to determine the presence or absence of *Vibrio* and another 25 g was used for the detection of *Aeromonas* as described by Mossel et al. (2003).

The preparation of the dilutions for *Vibrio* and *Aeromonas* inoculated samples was performed as in the case of MVC, PVC and Enterobacteriaceae count but decimal dilutions were made in alkaline peptone water and platted in duplicate on TCBS agar (Panreac, Barcelona, Spain) for *Vibrio* count and on *Aeromonas* medium base Ryan (Oxoid) for *Aeromonas* count. Plates were incubated for 24–48 h at 37 °C in the case of *Vibrio* and 24–48 h at 30 °C for *Aeromonas*. Biochemical tests were performed to confirm both pathogens and adjust the obtained count (data not shown).

2.4. Gas analyses

Headspace gas composition of control batches (not inoculated) was measured throughout the display time with a gas analyser (Witt-Gasetechnic, OXYBABY, O_2/CO_2, Witten, Germany) by determining the concentrations of CO_2 and O_2.

2.5. Statistical analyses

All data were statistically analysed by the general linear model (GLM) procedure of SPSS, version 14.0 (SPSS, 2005) and the significance of differences among samples at each day of sampling was determined by analysis of variance (ANOVA). Mean values and standard errors (SEs) are reported in the table. Differences were considered significant if $p \leq 0.05$.

3. Results and discussion

3.1. Head space gas composition

Gas analyses were made each sampling day in all the studied batches. Table 1 shows the CO_2 absorption rates observed both at 0 °C and 4 °C. The lowest CO_2 percentages inside packages were observed at the end of the storage period (day sixteen). An increase in the percentage of CO_2 absorbed along the storage period probably due to the solubilisation of this compound in the liquid muscle fraction until equilibrium is attained was observed (Ruiz-Capillas and Moral, 2001; Sivertsvik et al., 2002). At both temperatures (0 °C and 4 °C), 80/20 batches showed the highest absorption rates followed by 70/30 and finally 60/40 samples. Differences between absorption rates by temperature were also found. Samples kept at 0 °C, showed a greater diminution in the percentage of CO_2 than those observed at 4 °C for the same atmosphere. We could conclude that lower temperatures improve the absorption of CO_2. These results agreed with bibliography findings (Coles et al., 2003; ICMSF, 1980b). On the other side, air samples showed a slight production of CO_2 (negative results in Table 1) probably due to respiring and metabolic processes as a result of microbial growth and the permeability of the packaging materials used. The highest CO_2 increase appeared in air samples kept at 4 °C.

Initial O_2 values were, 0.20% ± 0.01 in MAP batches (residual O_2 in MAP trays) and 20.5% ± 0.03 in air samples and these concentrations remained fairly constant for all the storage periods without exceeding 1.9% in MAP batches (results not shown). The final percentage of O_2 inside the package was probably the result of microbial growth due to the use by bacteria for respiring processes (Debevere and Boskou, 1996). The headspace gas composition is dynamic, with CO_2 dissolving in the muscle and being formed by tissue and bacterial respiration with the consumption of O_2 as Gill (1996) and Ercolini et al. (2006) have described.

3.2. Microbial count in control batches

3.2.1. Mesophilic, psychrotrophic and Enterobacteriaceae viable count

Fig. 1 shows the behaviour of microbial counts. Microorganisms in all MAP samples kept at 0 °C showed difficulty to grow, especially enterobacteria with minimum values throughout the storage period. A slight growth was observed during the first four days for mesophiles but then, all MAP batches presented a count decrease specially the 70/30 and 80/20 batches although there were no significant differences between them. Psychrotrophic count remained fairly constant or even decreased in batches 80/20 and 70/30 probably due to the higher CO_2 concentration

Table 1
Percentages of CO_2 absorbed at 0 °C and 4 °C.

Atmospheres	0 °C	4 °C
60%CO_2/40%N_2	5.30 ± 0.2%	4.17 ± 0.7%
70%CO_2/30%N_2	9.90 ± 1.1%	7.90 ± 0.9%
80%CO_2/20%N_2	12.20 ± 2.0%	9.20 ± 1.1%
Air	−1.35 ± 0.7%	−5.00 ± 0.3%

Points represent mean values ± standard error from three samples. Values show the difference between the initial and the final (day sixteen of storage) percentage of CO_2 inside the packages when maximum absorption rates were observed.

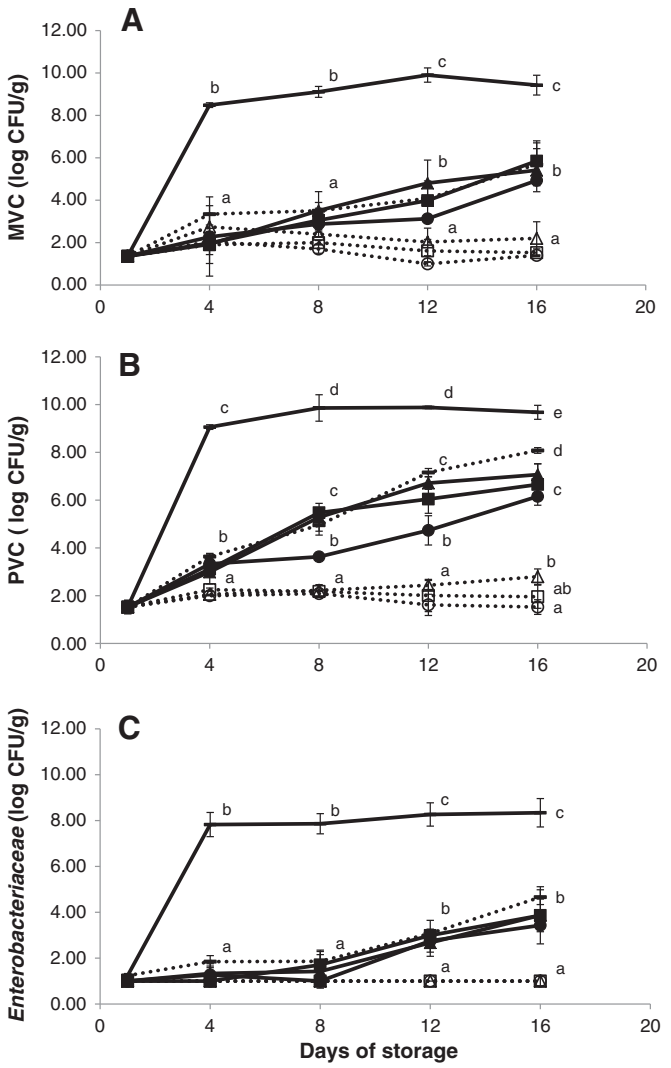

Fig. 1. Microbial growth in control batches (noninoculated). Points represent mean values ± standard error from three samples. Points of different MAP conditions taken at the same sampling time that are marked with the same letter are not significantly different ($p \leq 0.001$). A) Mesophilic viable count (MVC). B) Psychrotrophic viable count (PVC). C) Enterobacteriaceae count. Evolution at 0 °C: 60% CO_2/40% N_2 (··△··), 70% CO_2/30% N_2 (··□··), 80% CO_2/20% N_2 (··○··), air (··▬··). Evolution at 4 °C: 60% CO_2/40% N_2 (—▲—), 70% CO_2/30% N_2 (—■—), 80% CO_2/20% N_2 (—●—), air (——).

present in the package but 60/40 samples showed a slight growth until they reached final concentrations of 2.80 ±0.32 log CFU/g. The effect of CO_2 on microbial growth has been demonstrated by numerous authors being this effect greater as a result of increasing concentrations of carbon dioxide inside the packages (Kostaki et al., 2009; Torrieri et al., 2006; Provincial et al., 2010). Despite the lower storage temperature, air samples showed great growth for mesophiles exceeding the Spanish legal limit of 10^6 CFU/g (European Commission (EC), 2005) at day 16. Enterobacteriaceae count in air samples also exceeds the legal limit of 10^3 CFU/g since day 12.

On the other hand when samples were kept at 4 °C all microorganisms were able to grow, especially psychrotrophic ones. MVC increased throughout all the storage periods exceeding the legal limit of 10^6 CFU/g at day 16 in all MAP batches. Significant differences were found between MAP batches and air samples which exceeded the legal limit before day four. The same pattern was found in PVC although counts were higher in all batches. Significant differences were found between air and MAP batches as between MAPs presenting 80/20 samples as the lowest counts. Enterobacteria in MAP batches exceeded 10^3 CFU/g since day 12 and air samples since day 4.

Large variations in microbial counts are found between publications in terms of absolute values as well as time evolution due to the use of different temperatures, atmosphere combinations and cultivation procedures. The common conclusion of all these researches is that to achieve microbiological benefit, the storage temperature of MAP product should be as low as possible since solubility of CO_2 decreases with increase in temperature (Daniels et al., 1985) and also that MAP is effective in controlling microbial growth when compared with air packaging (Arashisar et al., 2004; Arkoudelos et al., 2007; Lysh et al.;, 2007; Noseda et al., 2012; Pantazi et al., 2008; Provincial et al., 2010).

3.2.2. Investigation of Vibrio and Aeromonas (presence/absence)

One hundred and twenty fillets were analysed to detect *Vibrio* and *Aeromonas* in noninoculated samples. All samples were negative for the investigation of *V. parahaemolyticus* although some suspicious colonies were found and tested biochemically. Incidence of *Vibrio* is seasonal dependent and it is directly related to the characteristics of the water where they develop and depends on numerous factors such as pH, salinity and temperature so the isolation is more common in temperate waters and in the summer (Duan and Su, 2005; Twedt, 1989). Therefore, it is not surprising not to find any positive results for *V. parahaemolyticus* on the analysed samples taking into account the location and environmental conditions of the fishing area and the season of fish catching (January–February 2010).

The sampling and analysis of all control samples (120 fillets) showed no positive results for *A. hydrophila*. *Aeromonas* growth is also affected by environmental factors. Counts are higher during the warmer seasons, when water temperature exceeds 20 °C whereas if temperatures are below 5 °C lower counts are observed. The failure to find any positive result to *A. hydrophila* in our samples is not surprising given the conditions in the area of capture, processing hygiene measures, capture station and mainly temperature of sample storage.

3.3. Microbial count in inoculated batches

3.3.1. Growth of V. parahaemolyticus

Fig. 2 shows the behaviour of *V. parahaemolyticus* count in inoculated samples at 0 °C or 4 °C for each tested strain. Microbial analyses at day one were carried out immediately after inoculating the packaging samples to verify the inoculum size.

Initial values of samples kept at 0 °C were 5.55 ± 0.31 log CFU/g and 5.58 ± 0.15 log CFU/g for V511 and V5304 respectively. A significant decrease of count in MAP samples could be observed although no significant differences were found between MAPs. At day sixteen of storage, 60/40 samples of V511 reach an inactivation of 2.91 log cycles, followed by 80/20 (3.50 log cycles) and 70/30 (3.29 log cycles). Strain V5304, showed slightly higher inactivation rates with values of 3.13, 3.46 and 3.67 log cycles for 60/40, 70/30 and 80/20 samples. Air samples inoculated with both strains of *Vibrio*, also presented a decrease in microbial counts. For both strains, significant differences were found between air and MAP samples, presenting these ones as greater inactivation. Final inactivation values at day sixteen were 1.96 log cycles for V511 and 1.25 log cycles for V5304.

MAP samples kept at 4 °C showed a similar pattern although inactivation values were lower. In this case, the storage temperature did not hide the effect of CO_2 and some significant differences were found between MAPs, showing batches with higher CO_2 concentration (80/20 and 70/30) as the lowest counts. For V511, maximum inactivation rates were 1.59, 2.52 and 2.68 log cycles for 60/40, 70/30 and 80/20 samples respectively and 1.71, 2.40 and 2.79 log cycles for strain 5304. On the other hand air samples presented significant growth probably due to the combination of a more proper storage temperature (4 °C) in combination with the absence of CO_2. V511 air samples showed an increase at the last day of storage of 3.25 log cycles and V5304 was able to grow 2.96 log cycles.

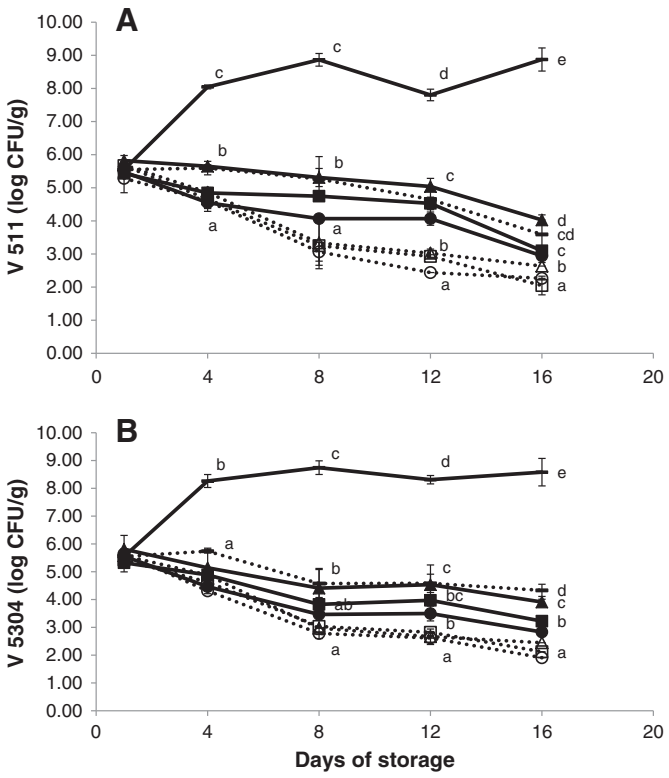

Fig. 2. *V. parahaemolyticus* count in inoculated batches. Points represent mean values ± standard error from three samples. Points of different MAP conditions taken at the same sampling time that are marked with the same letter are not significantly different ($p \leq 0.001$). A) Strain CECT 511. B) Strain CECT 5304. Evolution at 0 °C: 60% CO_2/40% N_2 (···△···), 70% CO_2/30% N_2 (···□···), 80% CO_2/20% N_2 (···○···), air (··▬···). Evolution at 4 °C: 60% CO_2/40% N_2 (—▲—), 70% CO_2/30% N_2 (—■—), 80% CO_2/20% N_2 (—●—), air (——).

Storage temperature is one of the most important factors to control microbial growth (Provincial et al., 2013). Kaneko and Colwell (1973) determined that the minimum *V. parahaemolyticus* growth temperature in natural matrices (food) was 10 °C but Beuchat (1973) observed *Vibrio* growth at 5 °C in TSB (trypticase soy broth) with alkaline pH. In a study by Saxena and Kulshrestha (1985) in fresh fish stored at temperatures between 0 °C and 4 °C, *V. parahaemolyticus* count fell more than 7 log units in 16 days as Muntada-Garriga et al. (1995) found. During the storage of oysters at 4 °C, a notable decrease in *Vibrio* count after a week of storage was found. Although low storage temperatures used did not get a total inactivation of *V. parahaemolyticus* a reduction in numbers could be observed and is in agreement with Ferreira-Magalhães et al. (2000) during storage of lobster meat for three months at 6 °C and Vasudevan et al. (2002) in the storage of fish fillets at 4 °C. Yang et al. (2008) also found a decrease in count of *V. parahaemolyticus* of approximately 3 log cycles during storage at 0 °C for 182 h in salmon fillets. The effect of storage temperature on the growth of *V. parahaemolyticus* has been deeply studied but the effect of CO_2 is still unclear. Kimura and Murakami (1993) studied the fate of some food pathogens in jack mackerel fillets stored at 5 °C under air and modified atmospheres. Their studies agree with our results and concluded that *V. parahaemolyticus* numbers decreased in all gas atmospheres tested but this reduction was larger in CO_2 batches than in air samples. Makino et al. (2011) also studied the growth depression of *V. parahaemolyticus* in shrimp stored at 20 °C under high CO_2 atmosphere (99%) when compared with air storage. Storage temperature enabled *V. parahaemolyticus* to grow but plate count of the tested strain, was clearly depressed by CO_2.

Differences between strains were also studied and it could be concluded that different *V. parahaemolyticus* strains have different resistance values to stressing factors as Kural et al. (2008) indicated.

3.3.2. Growth of A. hydrophila

A. hydrophila counts are shown in Fig. 3. Initial numbers were 5.32 log CFU/g and 5.21 log CFU/g for A5216 and A5734 at 0 °C. All MAP samples showed a count decrease although no significant differences were found between batches. Maximum inactivation values were 2.62 log cycles, 2.84 and 3.02 for A5216 packaged under 60, 70 and 80% of CO_2 respectively. Significant differences were found by comparing the strain effect. A5734, showed slightly higher inactivation rates for MAP batches presenting final numbers of 1.69, 1.49 and 1.51 log cycles for 60/40, 70/30 and 80/20 samples. Despite the low storage temperature used, air samples showed for both strains shorter inactivation rates statistically different from those observed in MAP batches. A5216 air samples showed a decrease of 0.63 log cycles at day sixteen while strain 5734 showed even a slight growth of 0.17 log cycles.

On the other hand, during the storage of sea bream fillets at 4 °C under air, 60/40 and 70/30 atm, *A. hydrophila* was able to grow. Significant differences were found between all atmospheres for both strains. Air samples showed the higher growth rate reaching final values of 3.46 and 1.82 log cycles for strain 5216 and 5734 at day sixteen followed by 60/40 samples (3.34 and 1.69 log cycles) and finally 70/30 fillets (2.31 and 0.12 log cycles). In contrast when sea bream fillets were packaged under 80% CO_2 concentration, growth inhibition was observed. *A. hydrophila* count decreased 0.22 log cycles for strain 5216 and 1.49 log cycles for A5734. Strain differences were noticed depending on the growing conditions. During the storage at 4 °C, A5216 showed higher growth rate than strain 5734 (and lower inactivation values for 80/20 samples) which could indicate that it was more resistant to the studied conditions. On the other hand, when storage temperature was lower, A5216 presented higher inactivation rates than A5734 so, it could be concluded that under more stressing conditions, A5216 seemed to be more sensitive than A5734.

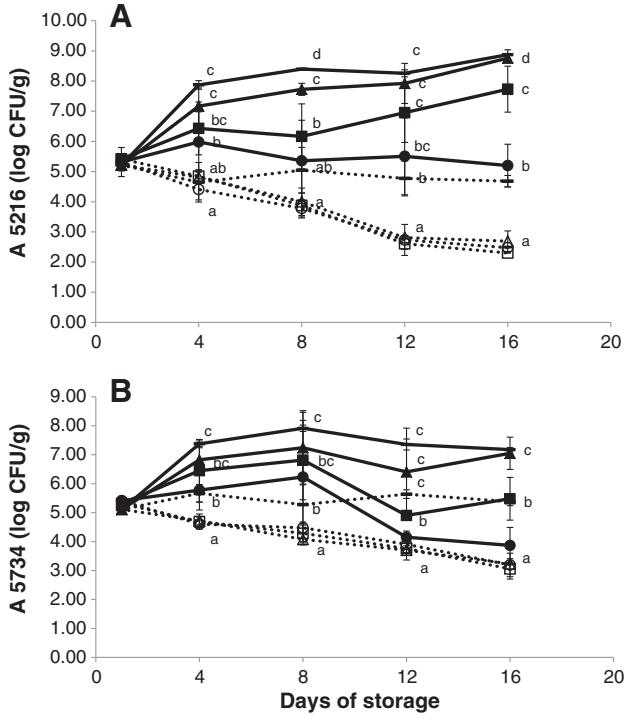

Fig. 3. *A. hydrophila* count in inoculated batches. Points represent mean values ± standard error from three samples. Points of different MAP conditions taken at the same sampling time that are marked with the same letter are not significantly different ($p \leq 0.001$). A) Strain CECT 5216. B) Strain CECT 5734. Evolution at 0 °C: 60% CO_2/40% N_2 (···△···), 70% CO_2/30% N_2 (···□···), 80% CO_2/20% N_2 (···○···), air (··—···). Evolution at 4 °C: 60% CO_2/40% N_2 (—▲—), 70% CO_2/30% N_2 (—■—), 80% CO_2/20% N_2 (—●—), air (——).

Considering the ubiquitous nature of *Aeromonas* spp. it is not surprising that large variations are found between isolates in terms of temperature tolerance as well as other factors affecting growth and survival (Merino et al., 1995). Rouf and Rigney (1971) found six of thirteen tested *A. hydrophila strains* able to grow at 0 °C, Gill and Reichel (1989) found growth even at −2 °C in vacuum beef packs and Lalitha and Surendran (2008) found an increase in *A. hydrophila* recovery during ice storage of prawns. These findings disagreed with our results, where *Aeromonas* stored at 0 °C was not able to grow (except in air samples). If storage at 0 °C may allow *A. hydrophila* growth, the observed reduction numbers could be attributed to the antimicrobial effect of CO_2 as well as the intrinsic factors of the sea bream muscle that differ from the models studied by the other authors. It is well known, that MAP enriched with CO_2 effectively inhibits growth of *A. hydrophila*, especially at low temperatures (Devlieghere et al., 2000; Ingham, 1990). Doherty et al. (1996), found *A. hydrophila* count decrease during the storage of lamb at 0 °C packaged under MAPs with 50 and 100% of CO_2. *Aeromonas* was also unable to grow during the storage of cooked crayfish tails for storage at 2 °C in packages with 80% of CO_2 although a moderate, slow growth was observed in air samples (Ingham, 1990). MAP (60% CO_2/40% O_2 and 70% CO_2/30% O_2) storage in conjunction with 0–2 °C affected the survival of *Aeromonas* spp. in pearlspot by reducing microbial load when compared with air packaging (Ravi Sankar et al., 2008). On the other hand, when samples were kept at 4 °C, CO_2 atmospheres were not efficient enough to control microbial growth except 80% CO_2 enriched atmospheres. These findings are in concordance with those of Bennik et al. (1995) where only atmospheres with 100% CO_2 concentrations were able to reduce *A. hydrophila* growth in culture broth at 8 °C and Doherty et al. (1996) who observed that *Aeromonas* was able to grow at 5 °C in lamb packaged with 20% and 50% CO_2 but not under 100% CO_2 concentrations. Golden et al. (1989) noticed that uninjured as well as heat injured *A. hydrophila* cells showed growth decrease in atmospheres with 100% CO_2 at 5 °C in contrast with bacterial growth under air conditions. From data in the literature it could therefore be concluded that growth of *A. hydrophila* is not completely stopped by CO_2 but is inhibited. Toxin production is one of the major risks associated with *A. hydrophila* presence. Storage at 4 °C enables *A. hydrophila* to produce cytotoxin, enterotoxin and hemolysin (Krovacek et al., 1991) in broth cultures. The use of enriched CO_2 modified atmospheres should be combined with strict maintenance of refrigeration temperatures as low as possible.

4. Conclusions

Although proper handling and cooking of fish products could reduce the risk of infection and illness, it is necessary to adopt commercial measures that guarantee the safety of fresh fish. The use of CO_2 enriched atmosphere packaging in combination with strict maintenance of temperatures of 0 °C ± 1 °C may reduce the microbial growth of both pathogens, *A. hydrophila* and *V. parahaemolyticus*, because, the lower the storage temperature, the greater the amount of absorbed CO_2 and therefore the greater antimicrobial effect achieved. During storage at 4 °C ± 1 °C, the antimicrobial effect of CO_2 became more evident because the great inhibitory effect of lower temperatures did not mask it. *A. hydrophila* and *V. parahaemolyticus* were able to grow in air packages during storage at 4 °C while the use of modified atmosphere allowed the reduction or even the inhibition of the growth of these pathogens. The antimicrobial effect of CO_2 is also more evident at higher concentrations being 80% the most efficient CO_2 concentration tested followed by 70% CO_2/30% N_2 atmosphere and finally 60% CO_2/40% N_2 combination. Strains of both pathogens had different sensitivities to temperature and CO_2. There are a lot of factors that could influence the effectiveness of CO_2 and temperature such as the strain, initial

inoculum load and indigenous flora count of the fillet. Further research would be needed to assess the real effect of these parameters on fresh gilthead sea bream fillets by testing different strains and varying the inoculum size.

Acknowledgements

This study was supported by the CENIT Acuisost project "Hacia una acuicultura sostenible" in collaboration with the Spanish fish company, Acuicultura Els Alfacs S.L.

References

Apromar, 2011. Apromar, the Marine Fish Aquaculture in Spain 2011. Available online: http://www.apromar.es/Informes/Informe-APROMAR-2011.pdf (Accessed 21.03.12).
Arashisar, S., Hisar, O., Kaya, M., Yanik, T., 2004. Effects of modified atmosphere and vacuum packaging on microbiological and chemical properties of rainbow trout (*Oncorynchus mykiss*) fillets. International Journal of Food Microbiology 97, 209–214.
Arkoudelos, J., Stamatis, N., Samaras, F., 2007. Quality attributes of farmed eel (*Anguilla anguilla*) stored under air, vacuum and modified atmosphere packaging at 0 °C. Food Microbiology 24, 728–735.
Bennik, M.H.J., Smid, E.J., Rombouts, F.M., Gorris, L.G.M., 1995. Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen. Food Microbiology 12, 509–519.
Beuchat, L.R., 1973. Interacting effects of pH, temperature and salt concentration on growth and survival of *Vibrio parahaemolyticus*. Applied Microbiology 25, 844–846.
Beuchat, L.R., 1982. *Vibrio parahaemolyticus*: public health significance. Food Technology 36, 80–83.
Broglia, A., Kapel, C., 2011. Changing dietary habits in a changing world: emerging drivers for the transmission of foodborne parasitic zoonoses. Veterinary Parasitology 182, 2–13.
CDC, 2009. *Vibrio parahaemolyticus*, General Information. National Center for Emerging and Zoonotic Infectious Diseases. Available online: http://www.cdc.gov/nczved/divisions/dfbmd/diseases/vibriop/ (accesed 05.05.12).
Chakraborty, R.D., Surendran, P.K., Joseph, T.C., 2008. Isolation and characterization of *Vibrio parahaemolyticus* from seafoods along the southwest coast of India. World Journal of Microbial Biotechnology 24, 2045–2054.
Coles, R., McDowell, D., Kirwan, M.J., 2003. Chapter 10: Modified Atmosphere Packaging. Food Packaging Technology. Blackwell Publishing. CRC Press, Florida, USA, pp. 304–311.
Daniels, J.A., Krishnamurthi, R., Rizvi, S.S.H., 1985. A review of effects of carbon dioxide on microbial growth and food quality. Journal of Food Protection 48, 532–537.
Daskalov, H., 2006. The importance of *Aeromonas hydrophila* in food safety. Food Control 17, 474–483.
Davies, A.R., Capell, C., Jehanno, D., Nychas, G.J.E., Kirby, R.M., 2001. Incidence of foodborne pathogens on European fish. Food Control 12, 67–71.
Debevere, J., Boskou, G., 1996. Effect of modified atmosphere packaging on the TVB/TMA-producing microflora of cod fillets. International Journal of Food Microbiology 31, 221–229.
Devlieghere, F., Lefevere, I., Magnin, A., Debevere, J., 2000. Growth of *Aeromonas hydrophila* in modified-atmosphere-packed cooked meat products. Food Microbiology 17, 185–196.
Doherty, A., Sheridan, J.J., Allen, P., McDowell, D.A., Blair, I.S., Harrington, D., 1996. Survival and growth of *Aeromonas hydrophila* on modified atmosphere packaged normal and high pH lamb. International Journal of Food Microbiology 28, 379–392.
Duan, J., Su, Y.C., 2005. Occurrence of *Vibrio parahaemolyticus* in two Oregon oyster-growing bays. Journal of Food Science 70, 58–63.
EPA. United States Environmental Protection Agency, 2006. *Aeromonas*: Human Health Criteria Document. Available online: http://water.epa.gov (accesed 19.04.12).
Epidemiological Surveillance System, 2007. Epidemiological comment on reported diseases and microbiological information system, Spain. Year 2006. Boletín Epidemiológico Semanal. , 15. Instituto de Salud Carlos III, MSC, pp. 109–114.
Ercolini, D., Russo, F., Torrieri, E., Masi, P., Villani, F., 2006. Changes in the spoilage-relates microbiota of beef during refrigerated storage under different packaging conditions. Applied and Environmental Microbiology 72, 4663–4671.
European, Commission (EC), 2005. Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Date of publication 22/12/2005.
European Commission Health, Protection Directorage-General, Consumer, 2001. Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on *Vibrio vulnificus* and *Vibrio parahaemolyticus* (in Raw and Undercooked Seafood) (Adopted on 19–20 September 2001).
FAO, 2010. Part 1. World review of fisheries and aquaculture. The State of World Fisheries and Aquaculture 2010, Rome, Italy, pp. 18–26.
Feldhusen, F., 2000. The role of seafood in bacterial foodborne diseases. Microbes and Infection 2, 1651–1660.
Ferreira-Magalhães, T., dos Fernandes, Silva, Vieira, R.H., Ferreira FaÇanha, S.H., Hofer, E., Martin, A.M., 2000. Growth of *Vibrio parahaemolyticus* in lobster homogenates at different temperatures. Food Science and Technology International 6, 145–150.
Gill, C.O., 1996. Extending the storage life of raw chilled meats. Meat Science 43, S99–S109.
Gill, C.O., Reichel, M.P., 1989. Growth of the cold-tolerant pathogens *Yersinia enterocolitica*. *Aeromonas hydrophila* and *Listeria monocytogenes* on high-pH beef packaged under vacuum or carbon dioxide. Food Microbiology 6, 223–230.
Golden, D.A., Eyles, M.J., Beuchat, L.R., 1989. Influence of modified-atmosphere storage on the growth of uninjured and heat-injured *Aeromonas hydrophila*. Applied and Environmental Microbiology 55, 3012–3015.
Hudson, J.A., Mott, S.J., Penney, N., 1994. Growth of *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Yersinia enterocolitica* on vacuum and saturated carbon dioxide controlled atmosphere-packaged sliced roast beef. Journal of Food Protection 57, 204–208.
ICMSF, 1980a. Pescados, mariscos y sus productos. In: Acribia, Zaragoza (Ed.), Ecología microbiana de los alimentos. , 2. Productos alimenticios, Spain, pp. 573–612.
ICMSF, 1980b. Los gases como conservadores. In: Acribia, Zaragoza (Ed.), Ecología microbiana de los alimentos. , 1. Factores que afectan a la supervivencia de los microorganismos en los alimentos, Spain, pp. 178–201.
Ingham, S., 1990. Growth of *Aeromonas hydrophila* and *Pleisomonas shigelloides* on cooked crayfish tails during cold storage under air, vacuum and a modified atmosphere. Journal of Food Protection 53, 665–667.
Jay, J.M., Loessner, M.J.A., Golden, D.A., 2009. Protección de los alimentos mediante atmósferas modificadas. Spain In: Zaragoza, Acribia (Ed.), Microbiología moderna de los alimentos, 5ª Edición. , pp. 355–358.
Johnson, W.G., Salinger, A.C., King, W.C., 1973. Survival of *Vibrio parahaemolyticus* in oyster shellstock at two different storage temperatures. Applied Microbiology 26, 122–123.
Kaneko, T., Colwell, R.R., 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. Bacteriology 113, 24–32.
Kimura, B., Murakami, M., 1993. Fate of food pathogens in gas-packaged jack mackerel fillets. Nippon Suisan Gakkaishi 59, 1163–1169.
Kostaki, M., Giatrakou, V., Savvaidis, I.N., Kontominas, M.G., 2009. Combined effect of MAP and thyme essential oil on the microbiological, chemical and sensory attributes of organically aquacultures sea bass (*Dicentrarchus labrax*) fillets. Food Microbiology 26, 475–482.
Krovacek, K., Faris, A., Månsson, I., 1991. Growth of and toxin production by *Aeromonas hydrophila* and *Aeromonas sobria* at low temperatures. International Journal of Food Microbiology 13, 165–175.
Kural, A.G., Shearer, A.E.H., Kingsley, D.H., Chen, H., 2008. Conditions for high pressure inactivation of *Vibrio parahaemolyticus* in oysters. International Journal of Food Microbiology 127, 1–5.
Lalitha, K.V., Surendran, P.K., 2008. Microbiological changes in farm reared freshwater prawn (*Macrobrachium rosenbergii* de Man) in ice. Food Control 17, 802–807.
Lin, C., Yu, R.C., Chou, C.C., 2004. Susceptibility of *Vibrio parahaemolyticus* to various environmental stresses after cold shock treatment. International Journal of Food Microbiology 92, 207–215.
Lysh, U., Lahtinen, J., Schelvis-Smit, R., 2007. Microbiological quality of maatjes herring stored in air and under modified atmosphere at 4 and 10 °C. Food Microbiology 24 (508–434), 516.
Makino, Y., Ito, Y., Oshita, S., Kawagoe, Y., Kawabuchi, Y., Sugawara, T., Hirata, T., 2011. Mathematical Analysis for Growth Depression of *Vibrio parahaemolyticus* in Shrimp Under a High Carbon Dioxide Atmosphere. Food Science and Technology Research 17, 63–67.
Martinez-Urtaza, J., Simental, L., Velasco, D., DePaola, A., Ishibashi, M., Nakaguchi, Y., Nishibuchi, M., Carrera-Flores, D., Rey-Alvarez, C., Pousa, A., 2005. Pandemic *Vibrio parahaemolyticus* O3:K6. Emerging Infectious Diseases 11, 1319–1320.
Merino, S., Rubires, X., Knøchel, S., Tomás, J.M., 1995. Emerging pathogens: *Aeromonas* spp. International Journal of Food Microbiology 28, 157–168.
Mossel, D.A.A., Moreno, B., Struijk, C.B., 2003. In: Acribia, Zaragoza (Ed.), Procedimientos normalizados y validados de trabajo para la comprobación o vigilancia en las industrias de alimentos y en la restauración colectiva. Microbiología de los alimentos, pp. 581–655.
Muntada-Garriga, J.M., Rodriguez-Jerez, J.J., Lopez-Sabater, E.I., Mora-Ventura, M.T., 1995. Effect of chill and freezing temperatures on survival of *Vibrio parahaemolyticus* inoculated in homogenates of oyster meat. Letters in Applied Microbiology 20, 225–227.
Noorlis, A., Ghazali, F.M., Cheah, Y.K., Tuan-Zainazor, T.C., Ponniah, J., Tunung, R., Tang, J.Y.H., Nishibuchi, M., Nakaguchi, Y., Son, R., 2011. Prevalence and quantification of *Vibrio* species and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level. International Food Research Journal 18, 689–695.
Noseda, B., Islam, M.T., Eriksson, M., Heyndrickx, M., De Reu, K., Van Langenhove, H., Devlieghere, F., 2012. Microbiological spoilage of vacuum and modified atmosphere packaged Vietnamese *Pangasius hypophthalmus* fillets. Food Microbiology 30, 408–419.
Pablos, M., Rodrígez-Calleja, J.M., Santos, J.A., Otero, A., García-López, M.L., 2009. Occurrence of motile *Aeromonas* in municipal drinking water and distribution of genes encoding virulence factors. International Journal of Food Microbiology 135, 158–164.
Pantazi, D., Papavergou, A., Pournis, N., Kontominas, M.G., Savvaidis, I.N., 2008. Shelf-life of chilled fresh Mediterranean swordfish (*Xiphias gladius*) stored under various packaging conditions: microbiological, biochemical and sensory attributes. Food Microbiology 25, 136–143.
Phillips, C.A., 1996. Review: modified atmosphere packaging and its effects on the microbiological quality and safety of produce. International Journal of Food Science and Technology 31, 463–479.
Provincial, L., Gil, M., Guillén, E., Alonso, V., Roncalés, P., Beltrán, J.A., 2010. Effect of modified atmosphere packaging using different CO_2 and N_2 combinations on physical, chemical, microbiological and sensory changes of fresh sea bass (*Dicentrarchus labrax*) fillets. International Journal of Food Science and Technology 45, 1828–1836.
Provincial, L., Guillén, E., Gil, M., Alonso, V., Roncalés, P., Beltrán, J.A., 2013. Survival of *Listeria monocytogenes* and *Salmonella enteritidis* in sea bream (*Sparus aurata*)

fillets packaged under enriched CO_2 modified atmospheres. International Journal of Food Microbiology 162, 213–219.
Ravi Sankar, C.N., Lalitha, K.V., Jose, Leema, Manju, S., Gopal, T.K.S., 2008. Effect of packaging atmospheres on the microbial attributes of pearlspot (*Etroplus suratensis* Bloch) stored at 0–2 °C. Food Microbiology 25, 518–528.
Rouf, M.A., Rigney, M.M., 1971. Growth temperatures and temperature characteristics of *Aeromonas*. Applied Microbiology 22, 503–506.
Ruiz-Capillas, C., Moral, A., 2001. Residual effect of CO_2 on hake (*Merluccius merluccius*) stored in modified and controlled atmospheres. European Food Research and Technology 212, 413–420.
Saxena, M.P., Kulshrestha, S.B., 1985. Effect of physicochemical factors on the survival of *Vibrio parahaemolyticus* on fish. Aquaculture 47, 369–372.
Sivertsvik, M., Jeksrud, W.K., Rosnes, J.T., 2002. A review of modified atmosphere packaging of fish and fishery products — significance of microbial growth, activities and safety. International Journal of Food Science and Technology 37, 107–127.
Torrieri, E., Cavella, S., Villani, F., y Masi, P., 2006. Influence of modified atmosphere packaging on the chilled shelf life of gutted farmed bass (*Dicentrarchus labrax*). J Food Engineering 77, 1078–1086.
Twedt, R.M., 1989. *Vibrio parahaemolyticus*. In: Doyle, M.P. (Ed.), Foodborne Bacterial Pathogens. Marcel Dekker Inc., Nueva York, USA, pp. 514–561.
Vasudevan, P., Marek, P., Daigle, S., Hoagland, T., Venkitanarayanan, K.S., 2002. Effect of chilling and freezing on survival of *Vibrio parahaemolyticus* of fish fillets. J. Food Safety 22, 209–217.
Yang, Z.Q., Jiao, X.A., Zhou, X.H., Cao, G.X., Fang, W.M., Gu, R.X., 2008. Isolation and molecular characterization of *Vibrio parahaemolyticus* from fresh, low-temperature preserved, dried and salted seafood products in two coastal areas of Eastern China. International Journal of Food Microbiology 125, 279–285.