Microbial Growth Models in Gilthead Sea Bream (Sparus Aurata) Stored in Ice

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Running title: Microbiological growth in sea bream

MICROBIAL GROWTH MODELS IN GILTHEAD SEA BREAM (*SPARUS AURATA*) STORED IN ICE

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Abstract

This study analyzes microbiological changes in whole, ungutted farmed gilthead sea bream (*Sparus aurata*) stored for an 18-day period in ice using traditional methods for mesophilic aerobic bacteria, psychrotrophic, *Pseudomonas* spp., *Aeromonas* spp., *Shewanella putrefaciens*, Enterobacteriaceae, sulphide-reducing *Clostridium* (Clostridia), and *Photobacterium phosphoreum* in muscle, skin, and gills, evaluating their seasonal differentiation. Two different statistical models were used to analyze microbiological growth. Simultaneously, physicochemical parameters such as the temperature, pH, biological oxygen demand (BOD_5), total dissolved solids, salinity, ammonia nitrogen, and total phosphorus content of growing waters were analyzed. The results showed that by the end of the storage time, specific spoilage bacteria (SSB) such as *Pseudomonas* spp., *Aeromonas* spp. and *S. putrefaciens* as H_2S-producing bacteria were dominant in sea bream harvested in temperate water in the Canary Islands. Muscle tissue had least contamination, followed by skin and gills. The values of the analyzed seawater parameters were constant during the four seasons, except that the temperature showed a small difference between winter and summer. Seasonal effects were observed among the fish analyzed, suggesting that the lower levels of contamination detected in winter may have been due to the slight difference observed in water temperature in that season.

Introduction

The determination of microbial growth and, thereby, fish shelf life with traditional microbiological tests is expensive and time-consuming (Bruckner et al., 2013). An alternative is the concept of predictive microbiology, which uses mathematical models to predict microbiological growth and, thus, to estimate the shelf life (McMeekin et al., 1993; Whiting, 1995). Predictive microbiology involves the development of mathematical models to describe the effect of the most important environmental factors controlling the responses of microorganisms in foods (Ross et al., 2000). The majority of these models are based on experimental data derived from laboratory media, and limited information is available for assessing individual cell variability in foods (François et al., 2006; Manios et al., 2011). The models have immediate practical application to improve microbial food safety and quality and also can provide quantitative understanding of the microbial ecology of foods (Ross et al., 2000).

In Spain, the farming of gilthead sea bream (*Sparus aurata*) has increased, with overall output rising from 127 tonnes in 1985 to 19430 tonnes in 2012. Although production has fallen by 15.9% over the previous year (2011), the Canary Islands are the third most important region in Spain for the production of gilthead sea bream (2740 tonnes in 2012), (APROMAR, 2013), mainly due to the ideal conditions of the ocean, namely temperature, salinity, nutrients, currents, and the morphology and nature of the seabeds (Pérez-Sánchez and Moreno-Batet, 1991). Water surface temperature in these islands ranges from 18ºC in winter to 22-23ºC in summer, an aspect that is important to take into consideration when studying the microbiology of farmed fish.

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Nevertheless, no studies have evaluated microbiology during the handling, distribution, and storage of this species harvested in the Canary Islands.

The growing production of this species of fish has increased the importance of maintaining their good quality during storage. Fish quality declines due to a complex process involving physical, chemical, and microbiological forms of deterioration. Enzymatic and chemical reactions are normally responsible for the initial loss of freshness, whereas microbial activity accounts for the obvious spoilage and thereby establishes product shelf-life (Guillén-Velasco et al., 2004; De Koning, 2004). Many factors can influence the rate of the microbial spoilage of fish, such as the bacterial flora present, storage conditions, handling, and temperature (Ward and Baj, 1988). Some bacterial groups are particularly associated with this spoilage, such as *Shewanella putrefaciens* and *Pseudomonas* spp., which are the specific spoilage bacteria (SSB) of iced fresh fish regardless of the origin of the fish (Gram and Huss, 1996). At room temperature, motile aeromonads are the specific spoilers of aerobically stored freshwater fish (Gorczyca and Pek Poh Len, 1985; Gram et al., 1990). Moreover, representatives of the families Vibrionaceae (Huss et al., 1995) and Enterobacteriaceae, lactic acid bacteria and yeasts (Koutsoumanis and Nychas, 1999; Koutsoumanis and Nychas, 2000) should also be considered. Modified atmosphere stored marine fish from temperate waters are spoiled by the CO_2 resistant *Photobacterium phosphoreum*, whereas Gram-positive bacteria are likely spoilers of CO_2 packed fish from fresh or tropical waters (Gram and Huss, 1996).

Clostridia are an important gastrointestinal pathogen associated with diarrheal disease of both humans and animals (Arroyo et al., 2005; Keel et al., 2007). Isolates that are indistinguishable

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from strains implicated in human disease can often be isolated from varying percentages of food and food producing animals. The presence of important *Clostridium difficile* strains in food sources has raised concerns about the potential for food-borne transmission (Rupnik, 2007).

Impedance is a microbiological method that can be used to determine bacterial counts within a short period of time (Koutsoumanis et al., 1999a). However, other methods such as polymerase chain reaction (PCR) have been used for the enumeration and identification of bacterial species (Boulares et al., 2011; Metcalf et al., 2011). In addition, traditional microbiological techniques have also been widely used for the identification and enumeration of different bacterial species in fish (Katikou et al., 2006; Özden et al., 2007; Baixas-Nogueras et al., 2009), including *S. putrefaciens* (Özden et al., 2007) and *P. phosphoreum* (Dalgaard et al., 1993; Dalgaard, 1995).

In recent years, several models have been developed to predict the growth of the different bacteria in fresh fish and fishery products (Dalgaard, 1995; Ross et al., 2000). The majority of the developed predictive mathematical models for chilled products describe the growth of SSB and pathogens based on the temperature, because this is the most important factor influencing shelf life (Zwietering et al., 1991; McMeekin et al., 1992; Gunvig et al., 2013). A two-step approach is employed: primary models are used to describe the growth of the SSB as a function of time, such as the modified Gompertz, Baranyi, and Roberts models or the Logistic model (Zwietering et al., 1990; Whiting, 1995; McDonald and Sun, 1999). Secondary models [e.g. the Arrhenius model, the Square root model (SRM), or response surface equations] are used to describe the temperature dependency of the growth of the SSB (Labuza and Riboh, 1982; Bratchell et al., 1990; Ross and McMeekin, 1994; Whiting, 1995, McDonald and Sun, 1999).

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But it is well-known that the prerequisite for the development of a successful predictive shelf life model requires sufficient knowledge of the spoilage process, the SSB itself, and the spoilage level, which determines the end of shelf life (Dalgaard et al., 1997, Koutsoumanis and Nychas, 2001).

The aim of this study was to develop a mathematical model that predicts growth of eight different bacteria in relation to seawater temperature in four different seasons and, moreover, to improve microbiological knowledge of gilthead sea bream (*Sparus aurata*) by determining the evolution of microorganisms responsible for spoilage and their counts in different tissues such as muscle, skin, and gills during iced storage.

Materials and methods

Gilthead sea breams, with an initial average weight of 490.8 g (420 g – 580 g), were obtained from an aquaculture farm located in Gran Canaria (Canary Islands, Spain; Atlantic Ocean, 27° 57′ 31″N, 15° 35′ 33″W). The fish were cultivated in floating cages, and four samplings were carried out: the first in winter (January), when average water temperature was 18 ºC (batch 1); the second in spring (April), 19.30 ºC (batch 2); the third in summer (June), 22 ºC (batch 3); and the fourth and last sampling in autumn (November), 21.30 ºC (batch 4); similar temperatures were described by Ginés et al. (2004). Fourteen fish per batch were sacrificed by immersion in iced fresh water (hypothermia), delivered to the laboratory within 1 hour of harvesting, and packed in polystyrene boxes with ice.

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On the day of slaughter (day 0 of the study), one whole ungutted fish was analyzed, while the other fish were kept in ice in polystyrene boxes with drainage holes. The ice was produced under hygienic conditions in an ice machine (ITV model IQ 135) and replenished when necessary. The fish were kept in a refrigerator with controlled temperature at 2 ± 1ºC. Further microbiological analyses were performed on days 2, 4, 7, 10, 14, and 18. Another fish was examined randomly in each analysis. Each sample was analyzed in duplicate; the results are the mean from both determinations.

Seawater samples were collected for four months, one sample per week, from two floating cages. In every unit, temperature, pH, salinity, total dissolved solids, and BOD_5 (APHA, 1992) were recorded using a Horiba U 22XD (Kyoto, Japan), which was placed in a depth of 1 m and at a distance of 8 m away from the cages, in order to avoid the direct influence of the fish discharges and the food residues. Seawater samples were also taken and analyzed for ammonia nitrogen and total phosphorus using the Agilent G1369A Spectrophotometer (Waldbronn, Germany).

Sample Preparation and Microbiological Analysis

Sea bream flesh (25 g/fish) was obtained aseptically from the dorsal anterior region of the right side of each fish, using the technique described by Slattery (1998). Sea bream skin (25 g) was removed aseptically from the central dorsal region of both sites of the fish. The samples were transferred to a Stomacher bag (Seward Medical, London, UK) containing 225 ml of 0.1% peptone water (Cultimed 413795) with salt (NaCl 0.85% w/v) (Drosinos and Nychas, 1996) and homogenized for 60 seconds using a Stomacher Lab Blender 400 at high speed (Stomacher, IUL

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Instruments, Barcelona, Spain). From these microbiological extracts, 9 decimal dilutions were prepared.

In addition to skin and flesh, gills were also analyzed. Gills from each fish were removed using sterilized scalpels and forceps (Cakli et al., 2006) and weighed (they had a weight approaching 10 g/fish), and the resulting value was multiplied by 9 to obtain the millilitres of the first serial dilution (Pascual and Calderón, 2002). No more than 30 minutes passed between dilution preparation and spreading on the appropriate media to avoid changes in the microbial population. Then, 1 ml of each one of the ten serial dilutions of three different tissues (skin, muscle, gills) was spread on the surface of each dry media except for the Enterobacteriaceae and Clostridia counts where the dilutions were introduced into molten agar.

Mesophilic bacteria were determined using Plate Count Agar (PCA Cultimed, 413799) incubated at 31 ºC for 72 h (Pascual and Calderón, 2002; ISO 4833:2003; Álvarez, et al., 2008; Corbo et al., 2008; Calanche et al., 2013; Genç et al., 2013). PCA was also used for psychrotrophic bacteria and incubated at 6.5 ºC for 7-10 days (technique proposed by ISO 17410, 2001; Broekaert et al., 2011). All colonies were counted on plates of countable dilutions. *Pseudomonas* spp. were determined by spreading on Pseudomonas F agar (Cultimed, 413796) incubated at 31ºC for 48 h and cream, fluorescent, or greenish colonies were counted. *Aeromonas* spp. were determined on BD *Yersinia Aeromonas* agar (BD, PA-25405605), after incubation at 31ºC for 48 h: pale colonies with a rose to red center and oxidase positive were counted.

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The counts of *Shewanella putrefaciens* (H$_2$S producing bacteria) were determined on Iron Agar Lyngby (IAL, prepared following indications and ingredients provided by OXOID). Iron agar plates were incubated at 20ºC for 48-72 h to count the black colonies formed by the production of H$_2$S (Dalgaard, 1995).

Photobacterium phosphoreum were also counted on Iron Agar Lyngby, with 0.1 ml being spread on a dried surface and incubated at 5ºC for 14 days. *P. phosphoreum* colonies appeared on the plates as transparent drops of dew (Dalgaard, 1995).\

Enterobacteriaceae were determined using Violet Red Bile Glucose Agar (VRBG) (Cultimed, 413745). From each dilution, 1 ml was inoculated into 10 ml of molten agar (45°C). After setting, a 10 ml overlay of molten medium was pour-platedapplied. Incubation was carried out at 37°C for 24 h and Enterobacteriaceaethese bacteria appeared as large colonies with purple haloes (Pascual and Calderon, 2002).

Clostridia (spores and vegetative cells) were determined on sulfite polymyxin sulfadiazine (SPS) (Cultimed, 414125); 1 ml of the dilutions was inoculated into tubes (15 ml) with molten medium (45ºC) and incubated at 46ºC for 24-48 hours. The black colonies observed in the tubes were multiplied by a dilution factor to obtain the number of CFU/g (Pascual and Calderón, 2002).

Counts were performed in duplicate and examined visually for typical colony types and morphology characteristics associated with each growth medium. The data were reported as colony forming units (log CFU/g). Conventional biochemical tests were carried out to ensure the

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final identification, and the strains were identified according to Barrow and Feltham (2004) (Table 1) and Smith Svanevik and Tore Lunestad (2011).

Statistical Analysis

For each bacteria, $N_{i,j,k,t}$ indicates the count (CFU/g) corresponding to ith fish, jth tissue (muscle, gill, skin), kth batch (seasons), and at the t observation day. In order to explore bacterial growth on the observation days, the $\log_{10} N_{i,j,k,t}$ was plotted against the day and by tissue (Figure 1) and batch (Figure 2). The plots for mesophiles, psychrotrophic, *Pseudomonas* spp., *S. putrefaciens, Aeromonas* spp., and Enterobacteriaceae suggested that the linear mixed-effects model should be used (Laird and Ware, 1982):

(Model 1) $\qquad \log_{10} N_{i,j,k,t} = \theta + fish_i + \alpha_j + \lambda_k + P_r(t) + e_{i,j,k,t}$

where $fish_i$ indicated the random effect of the fish ($i = 1,...56$), α_j the fixed tissue effect ($j = muscle, gill, skin$), λ_k the batch effect ($k = 1,2,3,4$), $P_r(t)$ is a polynomial of degree r (trend), and $e_{i,j,k,t}$ random error. The following formula was used: $\alpha_{muscle} = \lambda_1 = 0$ (muscle and batch 1 were the reference categories). It was assumed that the random effects $fish_i$ were independent random variables distributed according to $N(0, \sigma_f)$ and the error $e_{i,j,k,t}$, independent random variables $N(0, \sigma_e)$ and independent from the random effects $fish_i$. In order to determinate the optimum degree for the polynomial $P_r(t)$ (trend), we considered the degrees $r = 1, 2, 3$, and then

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the one that optimized the Akaike criterion (AIC) was selected. For all the bacteria, the optimum

degree obtained was $r = 2$, and, thus, $P_2(t) = \beta_1 \cdot t + \beta_2 \cdot t^2$ (the intercept is subsumed in θ). The

goodness of fit of the model was evaluated by the coefficient R^2, which measures the proportion

of the variability corresponding to the fixed effects of the model.

To account for excess zeros for the Clostridia and *P. phosphoreum* (44.6% for Clostridia

and 46.4% for *P. phosphoreum*), the zero-inflated Poisson (ZIP) model was defined as (Hall,

2000):

$$(\text{Model 2}) \quad \Pr\left(N_{i,j,k,t} = r \mid \mathbf{x}_{j,k,t}\right) = \begin{cases} \pi_t + \left(1 - \pi_t\right)\exp\left(-\mu_{j,k,t}\right) & ; \quad r = 0 \\[2mm] \left(1 - \pi_t\right)\dfrac{\exp\left(-\mu_{j,k,t}\right)\mu_{j,k,t}^r}{r!} & ; \quad r > 0 \end{cases}$$

This model consists of a combination of two distributions to incorporate extra zeros,

where $\ln \mu_{j,k,t} = \theta + \alpha_j + \lambda_k + \beta \cdot t$ and π_t was the probability of being an extra zero. The logistic

model $\text{logit}\,\pi_t = \gamma + \eta \cdot t$ (the zeros only depended on day t) was considered. Here, the vector

$\mathbf{x}_{j,k,t}$ summarized the covariate tissue (j), batch (k), and day (t). Note that the effects of tissue,

batch, and day are expressed with the same parameters for both models. All of the regression

models were fitted to our data using the R packages nlme (LME procedure) and PSCL (Zeroinfl

procedure).

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Results and Discussion

The investigation showed the general possibility to predict the expected growth values of eight microorganisms studied in sea bream stored in ice, in four different batches.

Models for the analysis of microbiological growth

Model 1

For all bacteria represented by model 1, the optimal polynomial trend obtained was the quadratic being all the coefficients β2 negatives, which means that the growth has a linear deceleration. Table 2 shows the coefficients R^2, all comparisons among tissues and among batches, and, finally, the coefficients β1 and β2 corresponding to the quadratic trends.

Figures 1 and 2 show the expected growth values of $\log_{10} N_{i,j,k,t}$ mesophilic aerobic bacteria, psychrotrophic bacteria, *Aeromonas* spp*., Pseudomonas* spp*.,* Enterobacteriaceae, and *S. putrefaciens* plotted against each observation day and according to tissue and batch, respectively. The Table 3 shows the mean of bacterial counts of four batches (P < 0.001). The linear trend for all log-counts over time justified the use of this model (1). Table 2 shows the estimates of the model parameters (SE) and all paired comparisons. For these microorganisms, spoilage levels in gills were higher than in skin and muscle (P < 0.001), with muscle being the least contaminated tissue. Almost all estimates of $\lambda_2 - \lambda_1$, $\lambda_3 - \lambda_1$ and $\lambda_4 - \lambda_1$ were significantly

greater than zero, with lower levels of spoilage corresponding to batch 1 (winter). Batch 4 was the most contaminated, except for Enterobacteriaceae and *S. putrefaciens*.

An effect over the batches was observed, supposedly due to the lower water temperature in winter (4 ºC lower) compared to summer, while the rest of the physicochemical values stood constant during the four studied seasons (Table 4). This was the determining factor for the growth of different types of microflora present in fish. According to Ward and Baj (1988), the microbiological condition of fish muscle is directly related to fishing ground and environmental factors. These results coincide with those published by Iliopoulou-Georgudaki et al. (2009), who observed that temperature, dissolved oxygen, and conductivity had a greater influence on microbiological populations in aquacultured fish. Other authors (Grigorakis et al., 2003, 2004) detected differences between microbial counts from December (water temperature, 14ºC) and July (water temperature, 25ºC), with results showing that summer fish presented higher rates of autolytic activity but lower rates of microbial spoilage.

Model 2

Figure 3 shows the counts $N_{i,j,k,t}$ of Clostridia and *P. Phosphoreum* over time according to tissue and per batch. Note that the excess of zeros for both counts suggested the use of model 2. The estimated values $\mu_{j,k,t}$ (expected growth) corresponding to this model and the estimated probabilities of zeros π_t are summarized in Table 5. The negative coefficients of the parameter η indicated that the probability of zeros for Clostridia and *P. phosphoreum* decreased over the observation period.

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The plot of these probabilities versus days (Figure 4) showed that the probability of zeros (no counts) decreased over time; from day 10, the probability of spoilage was near one.

Table 5 shows that the contamination levels for Clostridia and *P. phosphoreum* displayed significant differences (P < 0.001) between the three tissues sampled. Muscle was the least contaminated tissue, followed by skin and gills for Clostridia; skin was the least contaminated tissue for *P. phosphoreum*.

Significant differences (P < 0.001) were observed for the microorganisms among the four batches studied, except between batches 2-1 for *P. phosphoreum*. These results suggested that the irregular growth observed for the two bacteria was independent of parameters analyzed in this study. Growth delay may be due to the fact that Clostridia and *P. phosphoreum* can be the dominant cultivable bacterium in the gut tracts of some fish, such as hatchetfish, and it is a normal member of the gut flora of cod (*Gadus morua*) (Dalgaard et al., 1997).

Descriptive microbiological analysis

The data for microbial flora (log CFU/g) of aquacultured ungutted sea bream stored aerobically in ice at 2 ± 1 °C by tissue are shown in Table 3. The results are expressed as averages of the four batches sampled, allowing the comparison of these results with other similar studies.

All analyzed bacteria in the present study increased gradually throughout the storage period from day 0, except in the case of Clostridia and *P. phosphoreum*, which started to grow from days 4 and 7 in muscle, respectively. Mesophilic and psychrotrophic bacteria on the initial day (day 0)

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were 0.37 and not detected in muscle, 4.10 and 1.99 log CFU/g in skin, and 4.64 and 3.50 log CFU/g in gills, respectively. Similar results were obtained in muscle on days 0 and 3 (Grigorakis et al., 2003) or in skin on day 1 (Drosinos and Nychas, 1996) in ungutted sea bream stored in ice. However, other authors have reported higher results in the initial values of mesophilic and psychrotrophic bacteria in muscle of ungutted sea bream (Tejada and Huidobro, 2002; Lougovois et al., 2003; Kilinc et al., 2007; Özden et al., 2007), sea bream fillets (Erkan and Ueretener, 2010), and ungutted sea bass (Papadopoulos et al., 2003) or in skin of whole sea bream (Cakli et al., 2007; Erkan, 2007). The differences observed in mesophilic and psychrotrophic bacteria between the different studies may have been due to the microbiological conditions of fish muscle in ungutted sea bream, directly related to fishing ground, sanitary conditions of the slaughterhouse, and environmental factors (Ward and Baj, 1988).

Mesophilic and psychrotrophic bacteria counts reached 7 log CFU/g on days 10 and 18 in muscle, days 7 and 14 in skin, and days 4 and 7 in gill, respectively. This value was considered to be the maximum acceptability limit for fresh water and marine species as defined by the ICMSF (1986). Other authors studying sea bream muscle reported mesophilic and psychrotrophic values of 7.81 and 7.11 log CFU/g, respectively, after 21 days of storage (Álvarez et al., 2008) and 6.7 and 7 log CFU/g, after 16 and 13 days, respectively (Erkan and Ueretener, 2010), and 7 log CFU/g after 11 and 14 days, respectively, in different culture conditions (López-Caballero et al., 2002). Similar results were reported in skin with mesophilic and psychrotrophic counts of 7.20 and 7.35 log CFU/g after 15 days, respectively, and 6.6 and 6.8 log CFU/cm^2 after 13 days of storage, respectively (Cakli et al., 2007; Erkan, 2007). Furthermore, the mesophilic counts observed in fish in this study after 18 days were higher than

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the psychrotrophic counts (8.73 and 7.01 log CFU/g in muscle, 10.91 and 7.84 log CFU/g in skin, and 11.44 and 9.92 log CFU/g in gills, respectively).

Initial counts of SSB (specific spoilage bacteria: *Pseudomonas* spp*., S. putrefaciens*, as H_2S-producing bacteria, and *Aeromonas* spp.) were below the detection threshold (<1 log CFU/g) in muscle: 3.51, 1.10, and 3 log CFU/g in skin; 4.14, 2.31, and 3.65 log CFU/g in gills, respectively. Low counts of *Pseudomonas* spp. for ungutted European hake stored in ice were also reported by Baixas-Nogueras et al. (2009). Other authors described higher initial counts of *Pseudomonas* spp. in muscle, with 3.9 log CFU/g for sea bream (Özden et al., 2007) and 3.0 log CFU/g for sea bass (Papadopoulos et al., 2003; Paleologos et al., 2004), as well as 3.3 log CFU/g in gutted sardine samples (Erkan, 2008) and 2.88 log CFU/g in horse mackerel (Tzikas et al., 2007). Initial *S. putrefaciens* counts accounted for a large proportion of the microflora in muscle of several species such as: sea bream, with values of 4.4 log CFU/g (Özden et al., 2007); sea bass, with values of 2.2 log CFU/g (Paleologos et al., 2004); sardines, 3.3 log CFU/g (Erkan, 2008); and sea bream skin, 3.3 log CFU/g (Erkan, 2007). However, these values differed from those reported in our study, which coincided with those described by López-Caballero et al. (2002), Lougovois et al. (2003), and Baixas-Nogueras et al. (2009).

In general, *Pseudomonas* spp. was the dominant population on day 18 of storage, followed by *Aeromonas* spp. and *S. putrefaciens*, with values of 7.76, 7.49, and 8.05 log CFU/g in muscle; 10.11, 8.24, and 7.49 log CFU/g in skin; and 10.40, 9.02, and 8.05 log CFU/g in gills, respectively. Others authors have reported similar results in terms of *Pseudomonas* spp. and *S. putrefaciens* counts in muscle of sea bream, ranging from 6-7.8 log CFU/g (López-Caballero et

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al., 2002; Lougovois et al., 2003; Özden et al., 2007), sea bass, with 7-7.2 log CFU/g (*Pseudomonas* spp.), and 6.6 and 7 log CFU/g (*S. putrefaciens*) (Papadopoulos et al., 2003; Paleologos et al., 2004), as well as in sardines with values of 4 and 4.9 log CFU/g, respectively, after 9 days of storage (Erkan, 2008), horse mackerel with values of 6.42 and 5.12 log CFU/g, respectively, after 12 days of ice storage (Tzikas et al., 2007), and sea bream skin with 6.7 log CFU/g after 13 days of ice storage for H_2S-producing bacteria counts (Erkan, 2007).

Similar counts for *Pseudomonas* spp. and *S. putrefaciens* have been reported as for SSB in fish from temperate and tropical waters (Gillespie, 1981; Lima Dos Santos et al., 1981; Gram and Huss, 1996) and in fresh Mediterranean fish stored aerobically under refrigeration (Koutsoumanis and Nychas, 1999) or ice storage (Gennari and Tomaselli, 1988; Gennari et al., 1999; Sant'Ana et al., 2011). The values for *S. putrefaciens* reported in this study were lower than those observed for *Pseudomonas* spp. at the end of the storage period, possibly due to the fact that *Pseudomonas* spp. and *S. putrefaciens* have specific iron chelating systems (siderophores), and when these are grown in co-culture on fish samples, siderophore-producing *Pseudomonas* spp. inhibits the growth of *S. putrefaciens* (Gram and Dalgaard, 2002; Olafsdóttir et al., 2006).

Regarding *Aeromonas* spp., Nagar and Bandekar (2011) showed a few counts in Rohu (*Labeo rohita*) of 5.24 to 6.64 log CFU/g on day 0 and 7, respectively; both results were higher than those obtained in our study. However, most of the literature refers to the prevalence of this microorganism in the fish, not counts (CFU/g) (Galindo and Chopra, 2007), highlighting the importance of *A. hydrophila* as a pathogen conveyed through water (Daskalov, 2006).

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In this study, Enterobacteriaceae counts were lower than those of SSB at the end of storage, coinciding with the results reported for different fresh Mediterranean fish at the end of shelf life (Koutsoumanis et al., 1999b; Gennari et al., 1999; Tejada and Huidobro, 2002). Initial Enterobacteriaceae counts were not detected in muscle, 1.99 log CFU/g in skin, and 2.48 log CFU/g in gills, increasing to 5.19, 5.75, and 6.35 log CFU/g, respectively, after 18 days of ice storage. Initial Enterobacteriaceae counts in fresh fish muscle were similar to those reported for ungutted European hake (Baixas-Nogueras et al., 2009). Nevertheless, other authors have described higher initial counts, although these same authors have reported similar values to those described here after the period of ice storage in different species, such as sea bass with counts of 2 and 4.2 log CFU/g (initial and final counts, respectively) (Papadopoulos et al., 2003), sea bream with 3.9 and 5.6 log CFU/g (initial and final counts, respectively) (Özden et al., 2007), and sardines with 3.5 and 5.08 log CFU/g (initial and final counts, respectively) (Erkan, 2008). The contribution of Enterobacteriaceae to the microflora of fish and its spoilage potential must be taken into consideration especially in the case of polluted water or delay in chilling after catch (Chouliara et al., 2004), as well as in the filleting process (Moini et al., 2009). Although this group can grow at low temperatures, their abundance decreases during iced storage, possibly because their growth rate is lower than that of other Gram-negative psychrotrophic spoilers, making them poor competitors (Bahmani et al., 2011).

Initial counts of Clostridia and *P. phosphoreum* were not detected in all the tissues analyzed. However, these counts increased after 18 days of iced storage to 3.02 and 3.70 log CFU/g in muscle, 3.03 and 3.15 log CFU/g in skin, and 2.78 and 4.23 log CFU/g in gills, respectively. The counts and trend growth of these bacteria were different from others examined in the present

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study. Similar results were found in boque fish stored aerobically, where the contribution of *P. phosphoreum* was very small and relatively unimportant (Koutsoumanis et al., 1999b). Regarding Clostridia, the source of contamination could be a result of carriage by the fish, water contamination, or cross-contamination during processing and sale (Metcalf et al., 2011). Thus, in research carried out by Lalitha and Surendran (2002), the presence of Clostridia in fresh fish was described in 19% of the samples.

Conclusions

The mathematical models applied in the present study revealed that the lowest contamination was detected in muscle during winter. In addition, the final counts of SSB were similar to those obtained in fish from temperate and tropical waters in other studies. Since registered physicochemical parameters (except the temperature) remained relatively constant throughout the whole year, this could be the main factor for the observed seasonal difference. Future studies would offer more data about the influence of other physicochemical parameters over the microbiological evolution in ungutted sea bream in order to confirm whether the temperature is the determining factor.

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Table 1. Provisional identification of strains isolated from sea bream (*Sparus aurata*) stored in ice.

	Gram reactions	Morphology	Motility 15ºC	Oxidase	Catalase	HandL	TMAO	H$_2$S
Pseudomonas spp.	−	R	+	+	+	Ox	−	−
S. putrefaciens	−	R	+	+	+	-/Ox	+	+
Aeromonas spp.	−	R	+	+	+	F	±	±
P. phosphoreum	−	Cb*	±	−	+	F	±	−

Morphology: (cb) coccobacilli, (R) rods, (*) large round cells.

HandL: Oxidative or fermentative metabolism of glucose was performed in the medium of Hugh and Leifson (Hugh and Leifson, 1953).

TMAO: trimethylamine oxide (TMAO) reduction.

Table 2. Mixed model for microbial growth in sea bream for model (1).

	Mesophilic	Psychrotrophic	*Pseudomonas* spp.	*S. putrfaciens*	*Aeromonas* spp.	Entero-bacteriaceae
R^2	0.976	0.985	0.975	0.956	0.964	0.938
$\alpha_{gill} - \alpha_{muscle}$	3.12 (0.13)**	2.87 (0.08)**	2.78 (0.11)**	0.87 (0.13)**	2.22 (0.12)**	1.22 (0.11)**
$\alpha_{skin} - \alpha_{muscle}$	2.45 (0.13)**	0.91 (0.08)	2.30 (0.11)**	0.17 (0.14)	1.63 (0.12)**	0.73 (0.11)**
$\alpha_{gill} - \alpha_{skin}$	0.67 (0.13)**	1.95 (0.08)**	0.49 (0.11)**	0.71 (0.14)**	0.59 (0.12)**	0.49 (0.11)**
$\lambda_2 - \lambda_1$	0.59 (0.15)**	0.89 (0.10)**	0.62 (0.14)**	0.39 (0.16)	0.57 (0.14)**	0.27 (0.12)
$\lambda_3 - \lambda_1$	0.56 (0.15)**	0.69 (0.10)**	0.98 (0.14)**	0.71 (0.15)**	0.46 (0.14)**	0.11 (0.13)

$\lambda_4 - \lambda_1$	0.81 (0.15)**	0.94 (0.10)**	1.04 (0.14)**	0.13 (0.15)	0.98 (0.14)**	0.04 (0.12)
$\lambda_2 - \lambda_3$	0.03 (0.15)	0.20 (0.10)	-0.36 (0.14)	-0.32 (0.16)	0.11 (0.14)	0.15 (0.13)
$\lambda_2 - \lambda_4$	-0.22 (0.15)	-0.05 (0.10)	-0.42 (0.14)*	0.27 (0.16)	-0.41 (0.14)*	0.23 (0.12)
$\lambda_3 - \lambda_4$	-0.25 (0.15)	-0.25 (0.10)	-0.06 (0.14)	0.58 (0.15)**	-0.52 (0.14)**	0.07 (0.13)
β_1	0.60 (0.03)**	0.53 (0.02)**	0.56 (0.03)**	0.57 (0.03)**	0.52 (0.03)**	0.37 (0.03)**
β_2	-0.01 (0.002)**	-0.01 (0.001)**	-0.01 (0.001)**	-0.01 (0.002)**	-0.01 (0.002)**	-0.008 (0.001)**

(*) $P < 0.05$; (**) $P < 0.001$; all p-values correspond to a multiple linear comparison.

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Table 3. Changes in the bacterial count (log CFU/g) in the muscle (a), skin (b), and gills (c) of sea bream (*Sparus aurata*) stored in ice.

Bacteria Studied	Tissue	Days of storage in ice							P
		0	2	4	7	10	14	18	
	a	0.37 ± 0.74	2.62 ± 0.32	3.88 ± 0.33	5.02 ± 0.24	6.22 ± 0.28	7.50 ± 0.41	8.73 ± 0.39	<0.001
Mesophilic	b	4.10 ± 0.47	4.92 ± 0.66	5.96 ± 0.81	7.17 ± 0.82	8.79 ± 0.40	9.59 ± 0.55	10.91 ± 0.35	<0.001
	c	4.64 ± 0.46	5.54 ± 0.75	6.87 ± 0.65	7.93 ± 0.55	9.34 ± 0.31	10.38 ± 0.45	11.44 ± 0.58	<0.001
	a	N.D.	2.01 ± 0.24	3.07 ± 0.35	4.04 ± 0.33	5.06 ± 0.55	6.05 ± 0.30	7.01 ± 0.43	<0.001
Psychrotrophic	b	1.99 ± 0.31	2.83 ± 0.51	3.67 ± 0.58	4.69 ± 0.61	5.80 ± 0.48	6.80 ± 0.41	7.84 ± 0.46	<0.001
	c	3.50 ± 0.40	4.57 ± 0.44	5.81 ± 0.52	6.78 ± 0.42	7.81 ± 0.91	8.91 ± 0.69	9.92 ± 0.67	<0.001
Pseudomonas spp.	a	N.D.	2.53 ± 0.30	3.49 ± 0.49	4.63 ± 0.64	5.74 ± 0.64	6.81 ± 0.37	7.76 ± 0.50	<0.001

	b	3.51 ± 0.48	4.50 ± 0.43	5.48 ± 0.72	6.55 ± 0.81	7.82 ± 0.76	9.05 ± 0.85	10.11 ± 0.63	<0.001
	c	4.14 ± 0.39	5.17 ± 0.51	6.19 ± 0.59	7.06 ± 0.67	8.16 ± 0.63	9.30 ± 0.55	10.40 ± 0.51	<0.001
	a	N.D.	2.28 ± 0.26	3.40 ± 0.23	4.57 ± 0.47	5.82 ± 0.68	7.03 ± 0.48	8.05 ± 0.45	<0.001
S. putrefaciens	b	1.10 ± 0.74	2.83 ± 0.51	3.72 ± 0.33	4.60 ± 0.54	5.56 ± 0.55	6.68 ± 0.39	7.49 ± 0.44	<0.001
	c	2.31 ± 2.31	3.53 ± 0.40	4.45 ± 0.52	5.34 ± 0.74	6.33 ± 0.56	7.28 ± 0.38	8.05 ± 0.50	<0.001
	a	N.D.	2.23 ± 0.34	3.04 ± 0.19	4.24 ± 0.51	5.28 ± 0.53	6.42 ± 0.33	7.49 ± 0.43	<0.001
Aeromonas spp.	b	3.00 ± 0.12	4.00 ± 0.35	4.94 ± 0.59	5.71 ± 0.56	6.72 ± 0.58	7.52 ± 0.60	8.24 ± 0.72	<0.001
	c	3.65 ± 0.58	4.37 ± 0.56	5.39 ± 0.62	6.39 ± 0.56	7.33 ± 0.66	8.10 ± 0.54	9.02 ± 0.57	<0.001
	a	N.D.	2.22 ± 0.14	2.85 ± 0.18	3.30 ± 0.22	3.99 ± 0.27	4.67 ± 0.39	5.19 ± 0.38	<0.001
Enterobacteriaceae	b	1.99 ± 0.08	2.72 ± 0.22	3.34 ± 0.21	3.95 ± 0.12	4.50 ± 0.25	5.03 ± 0.10	5.75 ± 0.29	<0.001

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	c	2.48 ± 0.25	3.17 ± 0.27	3.59 ± 0.69	4.34 ± 0.16	5.34 ± 0.26	5.75 ± 0.29	6.35 ± 0.34 <0.001
	a	N.D.	N.D.	N.D.	0.25 ± 0.50	1.74 ± 0.38	2.38 ± 0.77	3.02 ± 0.59 <0.001
Clostridia	b	N.D.	N.D.	N.D.	0.83 ± 0.57	1.76 ± 0.32	2.31 ± 0.21	3.03 ± 0.46 <0.001
	c	N.D.	N.D.	0.40 ± 0.80	1.55 ± 0.33	2.03 ± 0.26	2.34 ± 0.24	2.78 ± 0.34 <0.001
	a	N.D.	N.D.	N.D.	N.D.	2.05 ± 0.51	2.93 ± 0.53	3.70 ± 0.53 <0.001
P. phosphoreum	b	N.D.	N.D.	N.D.	1.38 ± 0.92	2.32 ± 0.05	2.64 ± 0.09	3.15 ± 0.11 <0.001
	c	N.D.	N.D.	0.58 ± 0.68	2.51 ± 0.27	3.16 ± 0.18	3.64 ± 0.19	4.23 ± 0.20 <0.001

Mean of four batches ± standard deviation; $P < 0.001$

N.D. = not detected.

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Table 4. Mean values of seawater properties.

Parameters	January	April	June	November
Temperature (ºC)	18	19.30	22	21.30
pH	7.8	7.7	8.1	7.4
Salinity (g/l)	33	35.6	32.2	33.6
BOD_5 (mg/l)	<5	5.1	<5	<5
Total dissolved solids (TDS) (mg/l)	2.4	4.1	<2.0	3.4
Ammonia nitrogen (mg/l)	<0.1	0.9	<0.1	1.1
Total phosphorus (mg/l)	<1	<1	<1	<1

Table 5. Zero-inflated models with its Poisson and logistic parts for Clostridia and *P. phosphoreum* in sea bream stored in ice.

	Poisson part			Logistic part	
	Clostridia	*P. phosphoreum*		Clostridia	*P. phosphoreum*
$\alpha_{gill} - \alpha_{muscle}$	0.530 (0.040)**	0.976 (0.006)**	Intercept	7.519 (2.288)**	5.960 (1.576)**
$\alpha_{skin} - \alpha_{muscle}$	0.403 (0.042)**	-1.450 (0.0012)**	Day	-1.192 (0.346)**	-0.945 (0.241)**
$\alpha_{gill} - \alpha_{skin}$	0.127 (0.025)**	2.426 (0.011)**			
$\lambda_2 - \lambda_1$	0.293 (0.029)**	0.003 (0.008)			
$\lambda_3 - \lambda_1$	-0.438 (0.034)**	-0.292 (0.008)**			
$\lambda_4 - \lambda_1$	-0.924 (0.034)**	0.326 (0.007)**			

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$\lambda_2 - \lambda_3$	0.731 (0.037)**	0.295 (0.008)**
$\lambda_2 - \lambda_4$	1.217 (0.037)**	-0.323 (0.007)**
$\lambda_3 - \lambda_4$	0.486 (0.040)**	-0.618 (0.008)**
β (slope)	0.262 (0.004)**	0.350 (0.001)**

(*) $P < 0.05$; (**) $P < 0.001$; all p-values correspond to multiple linear comparison.

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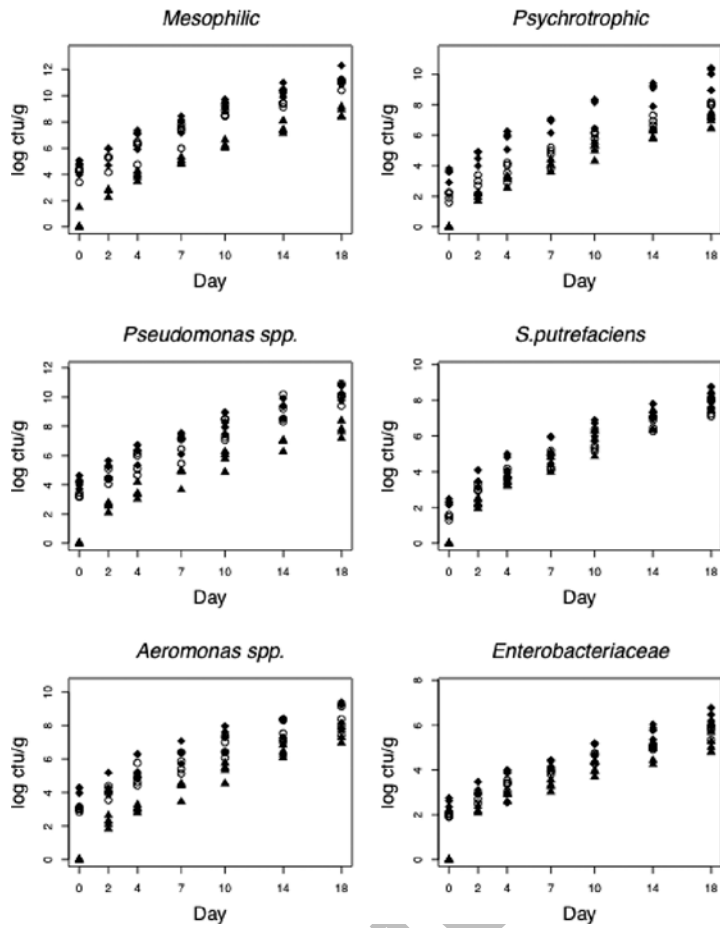Figure 1. Log counts of microorganisms (four batches) over time and according to tissue: ▲ = Muscle; ◆ = Gill; ○ = Skin.

Figure 2. Log counts of microorganisms (four batches) over time and per batch.
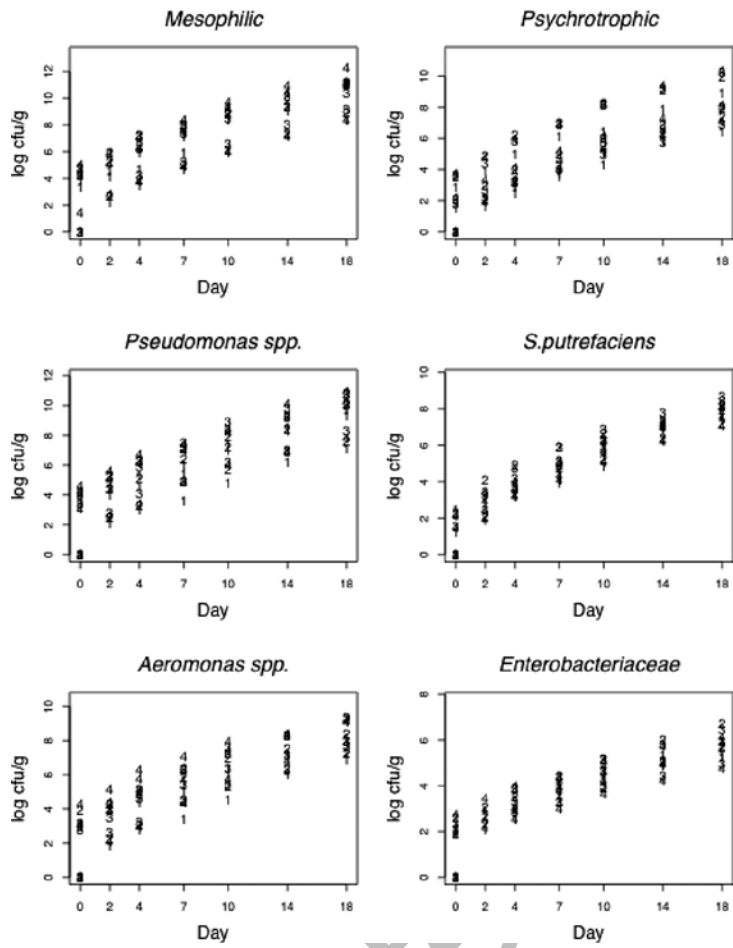
Figure 3. Counts of Clostridia and *P. phosphoreum* according to tissue and batch. The zero rates were 44.6% for Clostridia and 46.4% for *P. phosphoreum*.
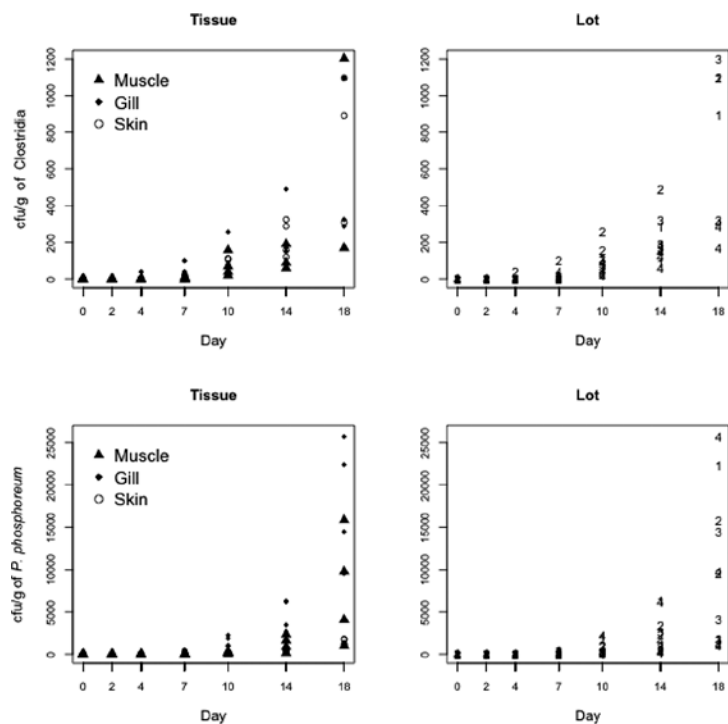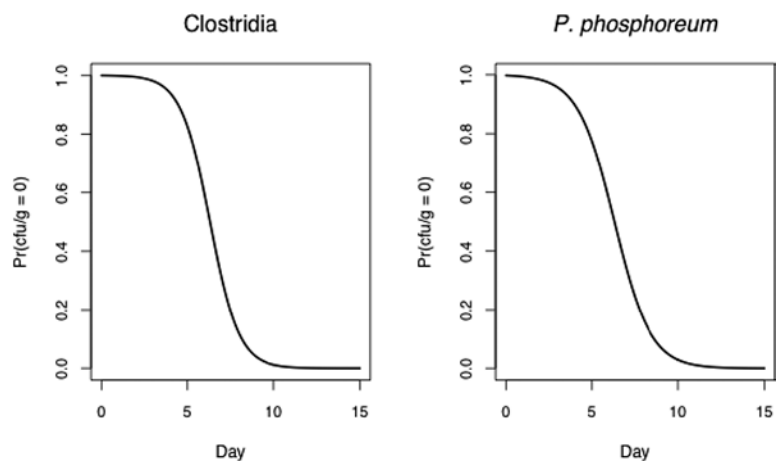

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Figure 4. Probabilities of zeros for Clostridia and *P. phosphoreum* by observation day.