Microbiological evolution of gilthead sea bream (*Sparus aurata*) in Canary Islands during ice storage

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Abstract

This study analyses the microbiological changes with traditional methods for total mesophilic aerobic, psychrotrophic, *Aeromonas* sp., *Pseudomonas* sp., *Shewanella putrefaciens*, Enterobacteriaceae, sulfide-reducers *Clostridium and Photobacterium phosphoreum* in muscle, skin and gills of whole ungutted gilthead sea bream (*Sparus aurata*) stored in ice during 18 days. The muscle tissue showed the minor grade of contamination, followed by the skin and the gills, with statistic significance ($p < 0.001$). The most prominent microorganisms in the different tissues and at the end of the storage were *Pseudomonas* sp. (7.76, 10.11 and 10.40 log CFU/g), *Aeromonas* sp. (7.49, 8.24 and 9.02 log CFU/g) and *S. putrefaciens*. (8.05, 7.49 and 8.05 log CFU/g) in sea bream harvested in the temperate water of the Canary Islands. The results obtained from this study can contribute to the improvement of 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.

Keywords: gilthead sea bream (*Sparus aurata*); ice storage; microbiological evolution; tissues.

Introduction

The demand in the European market for high-quality fresh fish stored in ice has increased in the last years, but the wide competition among producing countries in the Mediterranean area (Spain, Greece, Italy and Turkey) and consequent lowering of marked prices are demanding the differentiation and characterization of fish produced in aquaculture (Cakli *et al*., 2007).

In Spain, farming gilthead sea bream (*Sparus aurata*) has grown with an overall output rising from 127 tons in 1985 to 16360 tonnes in 2011. The Canary Islands are the third region of Spain in production of gilthead sea bream (3250 tonnes in 2011) (APROMAR, 2012), mainly due to their ideal oceanic conditions such as the nature of funds, salinity, nutrients, currents, and morphology, as well as the temperature (Pérez-Sánchez and Moreno-Batet, 1991). The superficial temperature of water in these islands oscillates between 18º C in Winter and 23º C in Summer, an aspect that is important to consider when studying the microbiology of farmed fish.

The increasing of production of these fish has raised the importance of keeping them in good conditions. The quality of the fish degrades due to a complex process in which physical, chemical, and microbiological forms of deterioration are implicated. Enzymatic and chemical reactions are usually responsible for the initial loss of freshness, whereas microbial activity is responsible for the obvious spoilage and its shelf life (Guillén-Velasco *et al*., 2004; de Koning *et al*., 2004). There are many factors that can influence on the rate of microbial spoilage of fish such as the bacterial flora present, the storage conditions, handling and temperature (Ward and Baj, 1988). Some bacterial groups are particularly

associated with this spoilage. Thus, the fish caught in cold marine waters and stored on ice under aerobic conditions show a spoilage that is usually dominated by *S. putrefaciens, Pseudomonas* sp., (Gram and Huss, 1996) and in less degree by the family *Vibrionaceae,* as well as Enterobacteriaceae, lactic acid bacteria and yeasts (Koutsoumanis and Nychas, 2000).

The aim of this study was to evaluate the microbiology during storage in ice of gilthead sea bream (*Sparus aurata*) harvested, carrying out the analysis of eight different microorganisms in three tissues, muscle, skin and gills. In the present work is included the count of the microbiota SSB (specific spoilage bacterial: *Pseudomonas* sp., *S. putrefaciens,* as H_2S-producing bacteria, and *Aeromonas* sp.) against other spoilage microorganisms. Encompassing this study a greater number of tissues and organisms, that hitherto published, thus contributing to a better understanding of microbial evolution in gilthead sea bream of aquaculture in temperate waters and allowing the development of a mathematical model which predicts the growth of eight different bacteria in future.

Materials and Methods

Fish Samples and Storage Conditions

Gilthead sea bream, 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). The fish were cultivated in two different floating cages and harvested in April. In this month four samplings were carried out consisted of 14 fish per cage, thus resulting in a total of 56 sea breams, which were sacrificed by immersion in ice water (hypothermia). The samples were delivered to the laboratory within 2 hours of harvesting and packaged

in polystyrene boxes with ice. On the initial day of the slaughter (day 0 of the study), two whole ungutted fish were analysed, whereas the rest of the samples were kept in polystyrene boxes with ice and holes to drain. The ice was produced under hygienic conditions in the ice machine (ITV model IQ 135), and replenished when necessary. At the laboratory, the fish were kept in boxes with ice and placed in a cold store refrigerator at 2.0 ± 1 ºC. Further microbiological analyses were performed during days 2, 4, 7, 10, 14 and 18. Randomly, other two fish were examined on each analysis. Each sample was analysed in duplicate, being the results the mean from both determinations.

The seawater samples were collected the days 1, 15 and 30 of April in two floating cages from an aquaculture unit located in the South of Gran Canaria. In both cages, temperature, pH, salinity, total dissolved solids and BOD_5 (APHA, 1992) were recorded using the Horiba U 22XD (Kyoto, Japan), which was placed in a depth of 1 m and in a distance of 8 m away from the cages to avoid the direct influence of the fish discharges and the food residues. Moreover, seawater samples were taken and analysed for ammonia nitrogen and total phosphorus using the Agilent G1369A Spectrophotometer (Waldbronn, Germany).

Sample Preparation and Microbiological Analysis

Sea bream skin and flesh (25 g of each sample) were obtained from the dorsal anterior region of the right side from each fish following the technique used by Slattery, (1988).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) as done by Drosinos and Nychas (1996), and homogenized for 60

s using a Lab Blender 400, Stomacher at high speed (Stomacher, IUL Instrument, Spain). The gills were also analysed and weighted (10 g/fish) and from this dilutions, other decimal dilutions were prepared.

Total viable counts (TVC) mesophilic and psychrotrophic bacteria were determined using Plate Count Agar (PCA Cultimed, 413799), and incubated at 31ºC for 72 hours and 5ºC for 7-10 days, respectively, as described by other authors (Pascual and Calderón, 2002; Broekaert *et al*., 2011). *Pseudomonas* sp. was enumerated on *Pseudomonas* F agar (Cultimed, 413796), incubated at 31ºC for 2 days and cream, fluorescent or greenish colonies were counted. *Aeromonas* sp. was determined on BD *Yersinia Aeromonas* agar (BD, PA-25405605), after incubation at 31ºC for 48 h and pale colonies with a rose to red centre and positive oxidase were counted.

The counts of *Shewanella putrefaciens* (H_2S producing bacteria) were determined on Iron Agar Lyngby (according indications and ingredients provided by OXOID). Iron agar plates were incubated at 20ºC for 48-72 h to enumerate the black colonies formed by production of H_2S, following the technique used by 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 (45°C). After setting, 10 ml overlay of molten medium was pour-plated. Incubation was done at 37 °C for 24 h and the bacteria were represented as large colonies with purple haloes as described by other authors (Pascual and Calderón, 2002).

Photobacterium phosphoreum were enumerated on Iron Agar Lyngb and the dilution (0.1 ml) was spread on dry surface and incubated at 5ºC for 14 days. These colonies appeared in the plates as transparent drops of dew (Dalgaard, 1995).

Sulfite-reducing *Clostridium* (clostridia), spores and vegetative cells, were determined on S.P.S. (Cultimed, 414125), one ml of the dilutions was inoculated into tubes (15 ml) with molten (45ºC) and incubated at 46ºC for 24-48 hours. The black colonies observed in the tubes were multiplied by a dilution factor as done by Pascual and Calderón (2002), to obtain the number of CFU/g.

Counts were performed in duplicate and examined for typical colony. The morphological characteristics were associated with each growth medium and the data were reported as colony forming units (log CFU/g). The conventional biochemical tests were carried out to ensure the final identification and the strains were identified according to Barrow and Feltham (1993) (Table 1) and Svanevik and Lunestad (2011).

Statistical analysis.

For each tissue and bacteria (mesophilic aerobic, psychrotrophic, *Aeromonas* sp., *Pseudomonas* sp., *S. putrefaciens* and Enterobacteriaceae) were obtained the means and standard deviations of the log-count of CFU/g for each day of observation. The log-counts were fitted against to the observation days using a model of linear regression. The linear trends were tested by means of the F-test for the linear regression. The counts of CFU/g of clostridia and *P. phosphoreum* showed a great number of zeros (39.3% for clostridia and *P. phosphoreum*). Therefore, for each observation day, these counts were resumed as medians and interquartile ranges (IQR), and fitted against to the observation day using a zero-inflated Poisson model. Statistical significance was set at p-value less than 0.001.

Results and Discussion

Physicochemical Data

Physicochemical data indicated a good chemical quality of the aquaculture's rearing seawater (Table 2), and moreover, they showed a great homogeneity in all samples taken. Although, April temperatures range from 18.7º to 19.5ºC.

Descriptive Microbiological analysis

The changes of the microflora of aquacultured ungutted sea bream during their storage in ice at 2.0º ± 1ºC are shown in Tables 3 and 4. Our results were expressed as an average (log CFU/g) or medians (log CFU/g) of each count on the fish analysed by daily control. The total viable counts (TVC) showed a gradual increase throughout the stored period from day 0, except for clostridia and *P. phosphoreum* that showed growth in muscle since days 4 and 7, respectively.

The TVC for mesophilic and psychrotrophic at the initial day (day 0) were 0.37 and no-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 determined in muscle at day 0 and 3 (Grigorakis *et al*. 2003) or in skin at day 1 (Drosinos and Nychas 1996) of ungutted sea bream stored in ice. However, other authors have reported higher results in the initial values of TVC 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 *et al*., 2010) and in ungutted sea bass (Papadopoulos *et al*., 2003) or in skin of

whole seabream (Cakli *et al*., 2007; Erkan, 2007). These differences observed on TVC could be due to the microbiological conditions of the fish muscle in ungutted sea bream, which are directly related to fishing ground, sanitary conditions of the slaughterhouse and environmental factors (Ward and Baj, 1988).

In reference to the mesophilic counts, they reached the value of 7 log CFU/g, on days 14 in muscle; 7 in skin, and 7 in gills, respectively. This value is considered as the maximum level for acceptability limit for freshwater and marine species International Commission on Microbiological Specifications for Foods (ICMSF, 1986). In addition, the psychrotrophic counts raised to 6 log CFU/g, on days 14 in muscle; 14 in skin and 7 in gills. This estimation of the psychrotrophic microorganisms gives better results to the shelf-life estimation of chilled fish than mesophilic bacteria and 6 log cfu/g could be accepted as the acceptability limit (Mol *et al*., 2007).

Other studies determined mesophilic and psychrotrophic values in muscle of the sea bream with 7.81 and 7.11 log CFU/g, after 21 days of the storage period (Álvarez *et al*., 2008) and 6.7 and 7 log CFU/g, after 16 and 13 days, (Erkan *et al*., 2010) and 7 log CFU/g after 11 and 14 days, respectively, using different culture conditions (López, *et al*., 2002). Similar results were determined in skin, with mesophilic and psychrotrophic counts of 7.20 and 7.35 log CFU/g after 15 days; and 6.6 and 6.8 log CFU/cm^2 after 13 days of storage, respectively (Cakli *et al*., 2007; Erkan *et al*., 2007). These results are similar to the mesophilic counts observed in our fish at 18 days, although they reached higher values than psychrotrophic (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 were below the detection threshold (<1 log CFU/g) in muscle; 3.51, 1.10 and 3 log CFU/g in skin; and 4.14, 2.31 and 3.65 log CFU/g in gills, respectively. Low counts of *Pseudomonas* sp. for ungutted european hake stored in

ice were also found by Baixas-Noguera *et al*. (2009). Other authors reported higher initial counts of *Pseudomonas* sp. in muscle, with 3.9 log CFU/g for sea bream (Özden *et al*., 2007), 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 samples of gutted sardine (Erkan *et al*., 2008) and 2.88 log CFU/g in horse mackerel (Tzikas *et al*., 2007). The initial *S. putrefaciens* counts constitute 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) and in sardines with 3.3 CFU/g (Erkan *et al*., 2008), as well as in sea bream skin where Erkan *et al*. (2007) reported 3.3 log CFU/g. However, these values were different to those reported in our study, which are in agreement with those observed by López *et al*., (2002), Lougovois *et al*. (2003) and Baixas-Noguera *et al*. (2009).

In general, *Pseudomonas* sp. was the dominant population on day 18 of storage, followed by *Aeromonas* sp. and *S. putrefaciens*, with values in muscle of 7.76, 7.49 and 8.05 log CFU/g; in skin of 10.11, 8.24 and 7.49 log CFU/g, and in gills of 10.40, 9.02 and 8.05 log CFU/g, respectively. Other authors have reported similar results for *Pseudomonas* sp. and *S. putrefaciens* counts in muscle of sea bream, ranging from 6-7.8 log CFU/g (López *et al*., 2002; Lougovois *et al*., 2003; Özden *et al*., 2007); sea bass with 7-7.2 log CFU/g (*Pseudomonas* sp.) and 6.6 and 7 log CFU/g (*S. putrefaciens*) (Papadopoulos *et al*., 2003; Paleologos *et al*., 2004*)*; as well as in sardines after 9 days of ice storage reached 4 and 4.9 log CFU/g, respectively (Erkan *et al*., 2008), horse mackerel, with 6.42 and 5.12 log CFU/g after 12 days of ice storage (Tzikas *et al*., 2007) and in sea bream skin, where was reported 6.7 log CFU/g after 13 days of ice storage for H_2S-producing bacteria counts (Erkan *et al*., 2007).

Similar counts for *Pseudomonas* sp. and *S. putrefaciens* have been reported as the SSB, regardless of the origin of the fish in temperate and tropical waters (Gillespie, 1981; Lima dos Santos, 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 of *S. putrefaciens* showed in our study were lower than those observed for *Pseudomonas* sp. at the end of the storage period, that could be due to *Pseudomonas* sp. and *S. putrefaciens* have specific iron chelating systems (siderophores), and when these bacteria grown in co-culture on fish samples siderophore, *Pseudomonas* sp. inhibits the growth of *S. putrefaciens* (Gram and Dalgaard, 2002; Olafsdóttir *et al*., 2006).

In our study, Enterobacteriaceae counts were lower to SSB on the final storage, which is in agreement with the results reported in different fresh Mediterranean fish at the end of the product's shelf life (Gennari *et al*., 1999; Koutsoumanis *et al*., 1999; Tejada and Huidobro, 2002). Therefore, the initial Enterobacteriaceae count was of <1 log CFU/g 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 iced storage. The initial count in fresh fish muscle was similar to that reported for ungutted european hake (Baixas-Noguera *et al*. 2009). Other authors have reported similar values in different species (initial and final counts), such as sea bass with counts of 2 and 4.2 log CFU/g (Papadopoulos *et al*., 2003), sea bream with 3.9 and 5.6 log CFU/g (Özden *et al*., 2007) and sardines with 3.5 and 5.08 log CFU/g (Erkan *et al*. 2008). The contribution of Enterobacteriaceae to the microflora of fish and its potential spoilage 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 of bacteria can grow at low temperatures, their abundance decreases during ice storage, possibly because their growth rate is lower than in others Gram-negative psychrotrophic spoilers (Bahmani *et al*., 2011).

Clostridia and *P. phosphoreum* initial counts were no detected in all the tissues analyzed. However, they increased after 18 days of iced storage to reach values of 1245 and 6923 CFU/g in muscle, 993 and 1456 CFU/g in skin, 710 and 18420 CFU/g, in gills, respectively. The counts and the trend growth of these bacteria were different to others examined in the present study. Similar results were found in boque fish stored aerobically, where the contribution of *P. phosphoreum* was extremely small and rather unimportant (Koutsoumanis *et al*. 1999).

Statistical Analysis

In Tables 3 and 4, the linear trend of bacterial counts (log CFU/g and CFU/g) in relation to the observation days and tissue was showed. This growth presented statistical significances ($p < 0.001$) between the different days sampled for all the studied bacteria. Thus, the Figures 1 and 2 showed the linear trend of both bacterial groups. The first group analysed had a real positive progression in all the tissues studied (Table 3), whereas in the second group (*Clostridium* and *P. phosphoreum*) there were an exponential progression since day 7 (Figure 1).

Conclusions

The results obtained from this study showed that the SSB were dominant in sea bream harvested in the temperate water of the Canary Islands, these were similar to those obtained in fish from temperate and tropical waters regarding to other studies. The statistical analysis revealed differences in the evolution of contamination (microbial growth) between the sampled tissues (muscle, skin and gills). Therefore, a thorough understanding of the spoilage process and knowledge of the specific spoilage organisms would be necessary to design a predictive mathematical model of the sea bream shelf life.

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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	H&L	TMAO	H_2S
Pseudomonas sp.	–	r	+	+	+	Ox	–	–
S. putrefaciens	–	r	+	+	+	-/Ox	+	+
Aeromonas sp.	–	r	+	+	+	F	±	±
P. phosphoreum	–	Cb*	±	–	+	F	±	–

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

H&L: 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. Mean values of physicochemical parameters of seawater.

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 3 Changes in the bacterial count (log CFU/g) according to tissue and observation day (mean±SD) in sea bream stored in ice.

	Tissue	0	2	4	7	10	14	18	P
Mesophilic	Muscle	0.37±0.74	2.62±0.32	3.88±0.33	5.02±0.24	6.22±0.27	7.50±0.41	8.73±0.39	<0.001
	Skin	4.10±0.47	4.92±0.67	5.96±0.81	7.17±0.81	8.78±0.40	9.59±0.55	10.91±0.34	<0.001
	Gills	4.64±0.46	5.54±0.74	6.87±0.64	7.93±0.55	9.34±0.31	10.38±4.64	11.44±0.57	<0.001
Psychrotrophic	Muscle	<1	2.01±0.23	3.07±0.34	4.04±0.32	5.05±0.54	6.05±0.3	7.01±0.42	<0.001
	Skin	1.99±0.30	2.83±0.51	3.67±0.57	4.69±0.60	5.80±0.48	6.80±0.40	7.84±0.46	<0.001
	Gills	3.50±0.40	4.57±0.43	5.81±0.51	6.78±0.42	7.81±0.90	8.91±0.69	9.92±0.67	<0.001
Enterobacteriaceae	Muscle	<1	2.22±0.14	2.85±0.18	3.30±0.22	3.98±0.27	4.67±0.39	5.19±0.38	<0.001
	Skin	1.99±0.08	2.72±0.22	3.34±0.21	3.95±0.12	4.50±0.24	5.03±0.09	5.75±0.28	<0.001
	Gills	2.47±0.25	3.17±0.27	3.59±0.69	4.34±0.16	5.04±0.26	5.75±0.28	6.35±0.34	<0.001
Pseudomonas sp.	Muscle	<1	2.53±0.29	3.49±0.49	4.63±0.64	5.73±0.61	6.81±0.36	7.76±0.49	<0.001
	Skin	3.51±0.47	0.50±0.42	5.48±0.72	6.55±0.80	7.81±0.76	9.05±0.84	10.11±0.62	<0.001
	Gills	4.14±0.39	5.17±0.50	6.19±0.59	7.06±0.67	8.15±0.62	9.30±0.55	10.40±0.50	<0.001
Aeromonas sp.	Muscle	<1	2.23±0.33	3.04±0.19	4.23±0.51	5.28±0.52	6.42±0.32	7.49±0.43	<0.001
	Skin	3.0±0.11	3.99±0.35	4.94±0.58	5.70±0.56	6.71±0.57	7.52±0.59	8.24±0.71	<0.001
	Gills	3.64±0.57	4.37±0.56	5.39±0.61	6.39±0.55	7.32±0.66	8.10±0.53	9.02±0.57	<0.001
S. putrefaciens	Muscle	<1	2.28±0.25	3.40±0.23	4.57±0.47	5.82±0.67	7.03±0.47	8.05±0.44	<0.001
	Skin	1.10±0.74	2.83±0.51	3.72±0.33	4.60±0.54	5.56±0.27	6.68±0.39	7.49±1.99	<0.001
	Gills	2.31±0.14	3.53±0.40	4.45±0.52	5.34±0.74	6.32±0.56	7.28±0.37	8.05±0.49	<0.001

All p-values correspond to multiple linear comparisons.

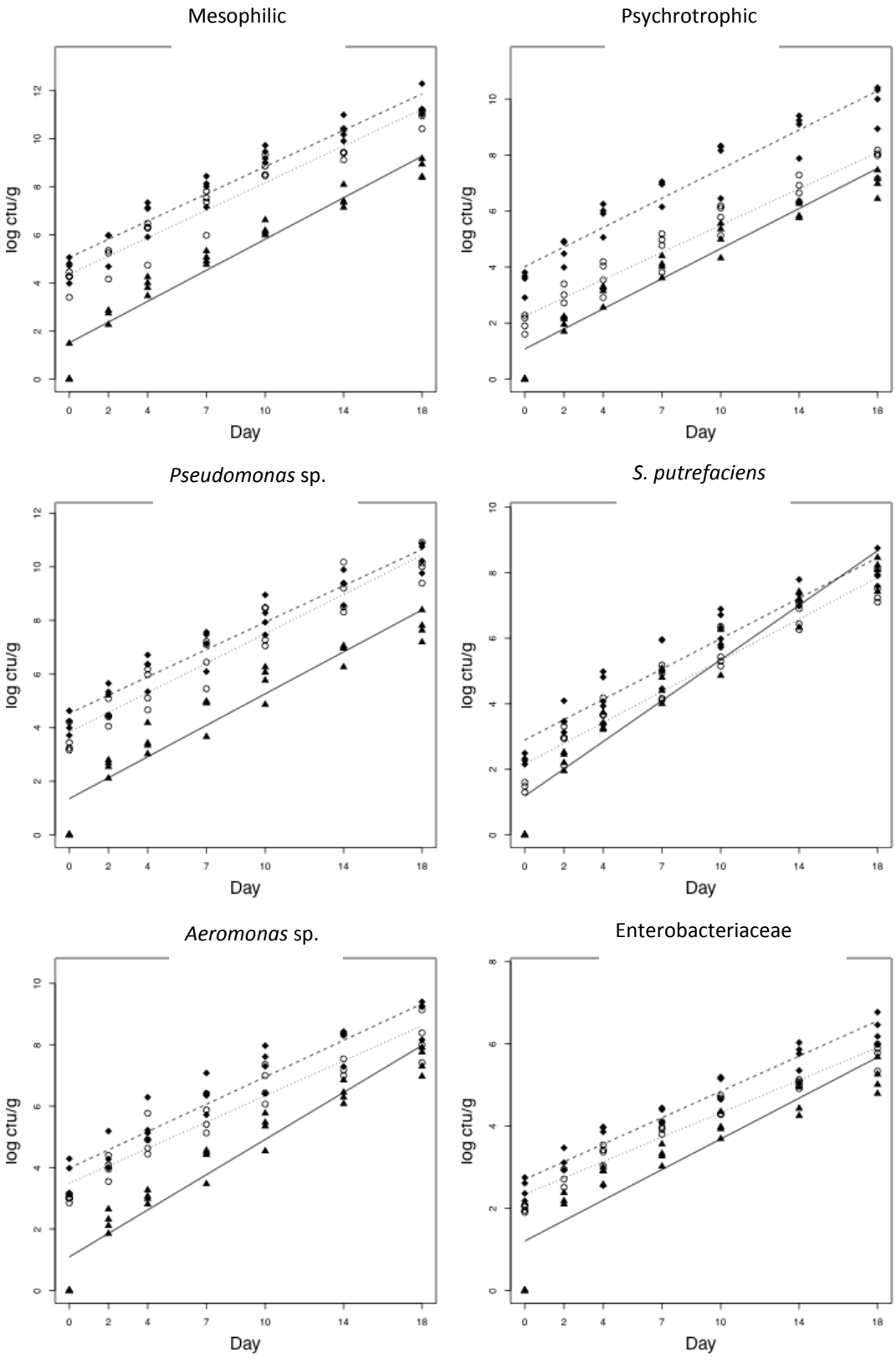
Table 4. Changes in Clostridia and *P. phosphoreum* counts (CFU/g) according to tissue and observation day (medians and interquartile ranges), in sea bream stored in ice.

	Tissue	0	2	4	7	10	14	18	P
Clostridia	Muscle	<1	<1	<1	<1 (0; 5)	54.50 (30; 113.5)	140 (74.5; 1640)	1245 (686; 2877)	<0.001
	Skin	<1	<1	<1	10 (5; 15)	70 (30; 110)	223 (139; 306)	993 (600; 2632)	<0.001
	Gills	<1	<1	<1 (0; 20)	30 (20; 70)	84 (75; 173)	176 (160; 336)	710 (306; 1192)	<0.001
P. Phosphoreum	Muscle	<1	<1	<1	<1	174 (74.5; 244)	1256.5 (519.5;1983)	6923 (2537;12810)	<0.001
	Skin	<1	<1	<1	60.5 (25; 80)	210 (186.5; 229)	437 (383;504)	1456 (1148.5; 1758)	<0.001
	Gills	<1	<1	5.0 (0;15)	403 (259; 465)	1454 (1001; 2050)	4816 (3048; 6238)	18420 (12002; 24045)	<0.001

P < 0.001; all p-values correspond to multiple linear comparison.

Figure 1. Log-CFU/g plotted against to observation day and its linear fitted;

▲——Muscle; ◆ ------ Gills; ○…… Skin.



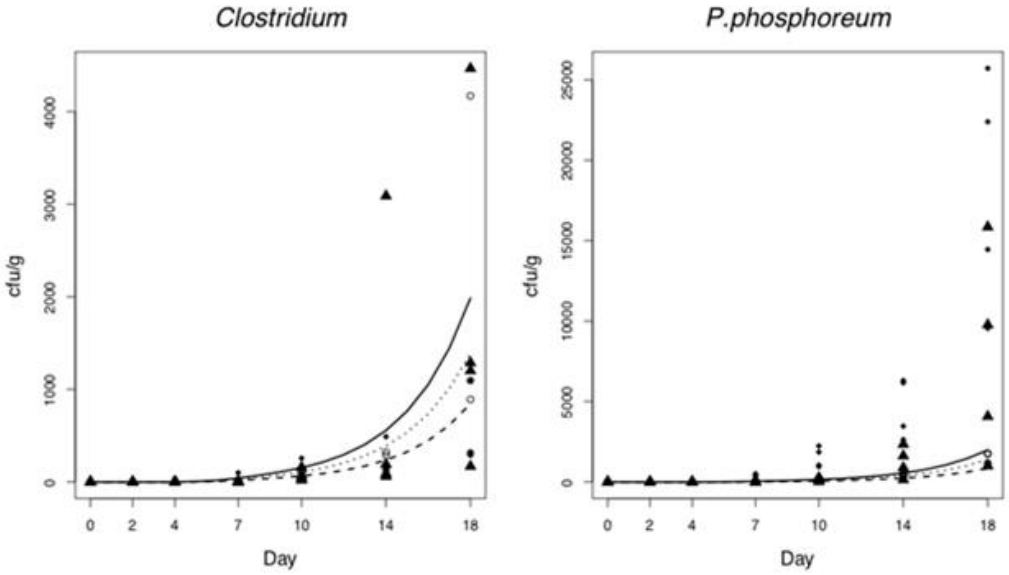


Figure 1. The cfu/g plotted against to the observation day and its fitted by means of a zero-inflated Poisson model. ▲ —— Muscle; ◆ ------ Gill; ○ ------ Skin

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