

1 **Microbiological evolution of gilthead sea bream (*Sparus aurata*) in Canary Islands**

2 **during ice storage**

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23

24 **Abstract**

25

26 This study analyses the microbiological changes with traditional methods for
27 total mesophilic aerobic, psychrotrophic, *Aeromonas* sp., *Pseudomonas* sp., *Shewanella*
28 *putrefaciens*, Enterobacteriaceae, sulfide-reducers *Clostridium* and *Photobacterium*
29 *phosphoreum* in muscle, skin and gills of whole ungutted gilthead sea bream (*Sparus*
30 *aurata*) stored in ice during 18 days. The muscle tissue showed the minor grade of
31 contamination, followed by the skin and the gills, with statistic significance ($p < 0.001$).
32 The most prominent microorganisms in the different tissues and at the end of the
33 storage were *Pseudomonas* sp. (7.76, 10.11 and 10.40 log CFU/g), *Aeromonas* sp. (7.49,
34 8.24 and 9.02 log CFU/g) and *S. putrefaciens*. (8.05, 7.49 and 8.05 log CFU/g) in sea
35 bream harvested in the temperate water of the Canary Islands. The results obtained from
36 this study can contribute to the improvement of microbiological knowledge of gilthead
37 sea bream (*Sparus aurata*) by determining the evolution of microorganisms
38 responsible for spoilage and their counts in different tissues such as muscle, skin, and
39 gills during iced storage.

40

41

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

44 **Introduction**

45

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

51 In Spain, farming gilthead sea bream (*Sparus aurata*) has grown with an overall
52 output rising from 127 tons in 1985 to 16360 tonnes in 2011. The Canary Islands
53 are the third region of Spain in production of gilthead sea bream (3250 tonnes in
54 2011) (APROMAR, 2012), mainly due to their ideal oceanic conditions such as
55 the nature of funds, salinity, nutrients, currents, and morphology, as well as the
56 temperature (Pérez-Sánchez and Moreno-Batet, 1991). The superficial
57 temperature of water in these islands oscillates between 18° C in Winter and 23° C
58 in Summer, an aspect that is important to consider when studying the
59 microbiology of farmed fish.

60 The increasing of production of these fish has raised the importance of keeping
61 them in good conditions. The quality of the fish degrades due to a complex
62 process in which physical, chemical, and microbiological forms of deterioration
63 are implicated. Enzymatic and chemical reactions are usually responsible for the
64 initial loss of freshness, whereas microbial activity is responsible for the obvious
65 spoilage and its shelf life (Guillén-Velasco *et al.*, 2004; de Koning *et al.*, 2004).

66 There are many factors that can influence on the rate of microbial spoilage of fish
67 such as the bacterial flora present, the storage conditions, handling and
68 temperature (Ward and Baj, 1988). Some bacterial groups are particularly

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

74 The aim of this study was to evaluate the microbiology during storage in ice of
75 gilthead sea bream (*Sparus aurata*) harvested, carrying out the analysis of eight
76 different microorganisms in three tissues, muscle, skin and gills. In the present
77 work is included the count of the microbiota SSB (specific spoilage bacterial:
78 *Pseudomonas* sp., *S. putrefaciens*, as H₂S-producing bacteria, and *Aeromonas* sp.)
79 against other spoilage microorganisms. Encompassing this study a greater number
80 of tissues and organisms, that hitherto published, thus contributing to a better
81 understanding of microbial evolution in gilthead sea bream of aquaculture in
82 temperate waters and allowing the development of a mathematical model which
83 predicts the growth of eight different bacteria in future.

84 **Materials and Methods**

85

86 ***Fish Samples and Storage Conditions***

87 Gilthead sea bream, with an initial average weight of 490.8 g (420 g–580 g), were
88 obtained from an aquaculture farm located in Gran Canaria (Canary Islands, Spain). The
89 fish were cultivated in two different floating cages and harvested in April. In this
90 month four samplings were carried out consisted of 14 fish per cage, thus resulting in a
91 total of 56 sea breams, which were sacrificed by immersion in ice water (hypothermia).
92 The samples were delivered to the laboratory within 2 hours of harvesting and packaged

93 in polystyrene boxes with ice. On the initial day of the slaughter (day 0 of the study),
94 two whole ungutted fish were analysed, whereas the rest of the samples were kept in
95 polystyrene boxes with ice and holes to drain. The ice was produced under hygienic
96 conditions in the ice machine (ITV model IQ 135), and replenished when necessary. At
97 the laboratory, the fish were kept in boxes with ice and placed in a cold store
98 refrigerator at 2.0 ± 1 °C. Further microbiological analyses were performed during days
99 2, 4, 7, 10, 14 and 18. Randomly, other two fish were examined on each analysis. Each
100 sample was analysed in duplicate, being the results the mean from both determinations.

101 The seawater samples were collected the days 1, 15 and 30 of April in two
102 floating cages from an aquaculture unit located in the South of Gran Canaria. In
103 both cages, temperature, pH, salinity, total dissolved solids and BOD₅ (APHA,
104 1992) were recorded using the Horiba U 22XD (Kyoto, Japan), which was placed
105 in a depth of 1 m and in a distance of 8 m away from the cages to avoid the direct
106 influence of the fish discharges and the food residues. Moreover, seawater
107 samples were taken and analysed for ammonia nitrogen and total phosphorus
108 using the Agilent G1369A Spectrophotometer (Waldbronn, Germany).

109

110 *Sample Preparation and Microbiological Analysis*

111

112 Sea bream skin and flesh (25 g of each sample) were obtained from the dorsal
113 anterior region of the right side from each fish following the technique used by
114 Slattery, (1988). The samples were transferred to a Stomacher bag (Seward Medical,
115 London, UK) containing 225 ml of 0.1% peptone water (Cultimed 413795) with salt
116 (NaCl 0.85% w/v) as done by Drosinos and Nychas (1996), and homogenized for 60

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

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

128 The counts of *Shewanella putrefaciens* (H₂S producing bacteria) were determined on
129 Iron Agar Lyngby (according indications and ingredients provided by OXOID). Iron
130 agar plates were incubated at 20°C for 48-72 h to enumerate the black colonies
131 formed by production of H₂S, following the technique used by Dalgaard (1995).

132 Enterobacteriaceae were determined using Violet Red Bile Glucose Agar (VRBG),
133 (Cultimed, 413745). From each dilution, 1 ml was inoculated into 10 ml of molten
134 (45°C). After setting, 10 ml overlay of molten medium was pour-plated. Incubation
135 was done at 37 °C for 24 h and the bacteria were represented as large colonies with
136 purple haloes as described by other authors (Pascual and Calderón, 2002).

137 *Photobacterium phosphoreum* were enumerated on Iron Agar Lyngb and the dilution
138 (0.1 ml) was spread on dry surface and incubated at 5°C for 14 days. These colonies
139 appeared in the plates as transparent drops of dew (Dalgaard, 1995).

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

145 Counts were performed in duplicate and examined for typical colony. The
146 morphological characteristics were associated with each growth medium and the data
147 were reported as colony forming units (log CFU/g). The conventional biochemical
148 tests were carried out to ensure the final identification and the strains were identified
149 according to Barrow and Feltham (1993) (Table 1) and Svanevik and Lunestad
150 (2011).

151

152 *Statistical analysis.*

153

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

164 **Results and Discussion**

165

166 ***Physicochemical Data***

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

170

171 ***Descriptive Microbiological analysis***

172

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

179 The TVC for mesophilic and psychrotrophic at the initial day (day 0) were 0.37 and
180 no-detected in muscle; 4.10 and 1.99 log CFU/g in skin, and 4.64 and 3.50 log
181 CFU/g in gills, respectively. Similar results were determined in muscle at day 0 and 3
182 (Grigorakis *et al.* 2003) or in skin at day 1 (Drosinos and Nychas 1996) of ungutted
183 sea bream stored in ice. However, other authors have reported higher results in the
184 initial values of TVC in muscle of ungutted sea bream (Tejada and Huidobro, 2002;
185 Lougovois *et al.*, 2003; Kilinc *et al.*, 2007; Özden *et al.*, 2007), sea bream fillets
186 (Erkan *et al.*, 2010) and in ungutted sea bass (Papadopoulos *et al.*, 2003) or in skin of

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

191 In reference to the mesophilic counts, they reached the value of 7 log CFU/g, on days
192 14 in muscle; 7 in skin, and 7 in gills, respectively. This value is considered as the
193 maximum level for acceptability limit for freshwater and marine species International
194 Commission on Microbiological Specifications for Foods (ICMSF, 1986). In addition,
195 the psychrotrophic counts raised to 6 log CFU/g, on days 14 in muscle; 14 in skin and 7
196 in gills. This estimation of the psychrotrophic microorganisms gives better results to the
197 shelf-life estimation of chilled fish than mesophilic bacteria and 6 log cfu/g could be
198 accepted as the acceptability limit (Mol *et al.*, 2007).

199 Other studies determined mesophilic and psychrotrophic values in muscle of the sea
200 bream with 7.81 and 7.11 log CFU/g, after 21 days of the storage period (Álvarez *et al.*,
201 2008) and 6.7 and 7 log CFU/g, after 16 and 13 days, (Erkan *et al.*, 2010) and 7 log
202 CFU/g after 11 and 14 days, respectively, using different culture conditions (López, *et*
203 *al.*, 2002). Similar results were determined in skin, with mesophilic and psychrotrophic
204 counts of 7.20 and 7.35 log CFU/g after 15 days; and 6.6 and 6.8 log CFU/cm² after 13
205 days of storage, respectively (Cakli *et al.*, 2007; Erkan *et al.*, 2007). These results are
206 similar to the mesophilic counts observed in our fish at 18 days, although they reached
207 higher values than psychrotrophic (8.73 and 7.01 log CFU/g in muscle; 10.91 and 7.84
208 log CFU/g in skin and 11.44 and 9.92 log CFU/g in gills, respectively).

209 Initial counts of SSB were below the detection threshold (<1 log CFU/g) in muscle;
210 3.51, 1.10 and 3 log CFU/g in skin; and 4.14, 2.31 and 3.65 log CFU/g in gills,
211 respectively. Low counts of *Pseudomonas* sp. for ungutted european hake stored in

212 ice were also found by Baixas-Noguera *et al.* (2009). Other authors reported higher
213 initial counts of *Pseudomonas* sp. in muscle, with 3.9 log CFU/g for sea bream
214 (Özden *et al.*, 2007), 3.0 log CFU/g for sea bass (Papadopoulos *et al.*, 2003;
215 Paleologos *et al.*, 2004), as well as 3.3 log CFU/g in samples of gutted sardine
216 (Erkan *et al.*, 2008) and 2.88 log CFU/g in horse mackerel (Tzikas *et al.*, 2007). The
217 initial *S. putrefaciens* counts constitute a large proportion of the microflora in muscle
218 of several species such as sea bream, with values of 4.4 log CFU/g (Özden *et al.*,
219 2007); sea bass with values of 2.2 log CFU/g (Paleologos *et al.*, 2004) and in
220 sardines with 3.3 CFU/g (Erkan *et al.*, 2008), as well as in sea bream skin where
221 Erkan *et al.* (2007) reported 3.3 log CFU/g. However, these values were different to
222 those reported in our study, which are in agreement with those observed by López *et*
223 *al.*, (2002), Lougovois *et al.* (2003) and Baixas-Noguera *et al.* (2009).

224 In general, *Pseudomonas* sp. was the dominant population on day 18 of storage,
225 followed by *Aeromonas* sp. and *S. putrefaciens*, with values in muscle of 7.76, 7.49
226 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,
227 9.02 and 8.05 log CFU/g, respectively. Other authors have reported similar results for
228 *Pseudomonas* sp. and *S. putrefaciens* counts in muscle of sea bream, ranging from 6-
229 7.8 log CFU/g (López *et al.*, 2002; Lougovois *et al.*, 2003; Özden *et al.*, 2007); sea
230 bass with 7-7.2 log CFU/g (*Pseudomonas* sp.) and 6.6 and 7 log CFU/g (*S.*
231 *putrefaciens*) (Papadopoulos *et al.*, 2003; Paleologos *et al.*, 2004); as well as in
232 sardines after 9 days of ice storage reached 4 and 4.9 log CFU/g, respectively (Erkan
233 *et al.*, 2008), horse mackerel, with 6.42 and 5.12 log CFU/g after 12 days of ice
234 storage (Tzikas *et al.*, 2007) and in sea bream skin, where was reported 6.7 log
235 CFU/g after 13 days of ice storage for H₂S-producing bacteria counts (Erkan *et al.*,
236 2007).

237 Similar counts for *Pseudomonas* sp. and *S. putrefaciens* have been reported as the
238 SSB, regardless of the origin of the fish in temperate and tropical waters (Gillespie,
239 1981; Lima dos Santos, 1981; Gram and Huss, 1996), and in fresh Mediterranean
240 fish stored aerobically under refrigeration (Koutsoumanis and Nychas, 1999) or ice
241 storage (Gennari and Tomaselli, 1988; Gennari *et al.*, 1999; Sant'Ana *et al.*, 2011).
242 The values of *S. putrefaciens* showed in our study were lower than those observed
243 for *Pseudomonas* sp. at the end of the storage period, that could be due to
244 *Pseudomonas* sp. and *S. putrefaciens* have specific iron chelating systems
245 (siderophores), and when these bacteria grown in co-culture on fish samples
246 siderophore, *Pseudomonas* sp. inhibits the growth of *S. putrefaciens* (Gram and
247 Dalgaard, 2002; Olafsdóttir *et al.*, 2006).

248 In our study, Enterobacteriaceae counts were lower to SSB on the final storage,
249 which is in agreement with the results reported in different fresh Mediterranean fish
250 at the end of the product's shelf life (Gennari *et al.*, 1999; Koutsoumanis *et al.*, 1999;
251 Tejada and Huidobro, 2002). Therefore, the initial Enterobacteriaceae count was of
252 <1 log CFU/g in muscle; 1.99 log CFU/g in skin and 2.48 log CFU/g in gills,
253 increasing to 5.19, 5.75, and 6.35 log CFU/g, respectively, after 18 days of iced
254 storage. The initial count in fresh fish muscle was similar to that reported for
255 ungutted european hake (Baixas-Noguera *et al.* 2009). Other authors have reported
256 similar values in different species (initial and final counts), such as sea bass with
257 counts of 2 and 4.2 log CFU/g (Papadopoulos *et al.*, 2003), sea bream with 3.9 and
258 5.6 log CFU/g (Özden *et al.*, 2007) and sardines with 3.5 and 5.08 log CFU/g (Erkan
259 *et al.* 2008). The contribution of Enterobacteriaceae to the microflora of fish and its
260 potential spoilage must be taken into consideration especially in the case of polluted
261 water or delay in chilling after catch (Chouliara, *et al.*, 2004), as well as in the

262 filleting process (Moini *et al.*,2009). Although this group of bacteria can grow at low
263 temperatures, their abundance decreases during ice storage, possibly because their
264 growth rate is lower than in others Gram-negative psychrotrophic spoilers (Bahmani
265 *et al.*, 2011).

266 Clostridia and *P. phosphoreum* initial counts were no detected in all the tissues
267 analyzed. However, they increased after 18 days of iced storage to reach values of
268 1245 and 6923 CFU/g in muscle, 993 and 1456 CFU/g in skin, 710 and 18420 CFU/g,
269 in gills, respectively. The counts and the trend growth of these bacteria were different
270 to others examined in the present study. Similar results were found in boque fish
271 stored aerobically, where the contribution of *P. phosphoreum* was extremely small
272 and rather unimportant (Koutsoumanis *et al.* 1999).

273

274 ***Statistical Analysis***

275

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

283

284 **Conclusions**

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

293

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295

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297

298 **References**

299

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

	Gram reactions	Morphology	Motility 15°C	Oxidase	Catalase	H&L	TMAO	H ₂ S
<i>Pseudomonas</i> sp.	–	r	+	+	+	Ox	–	–
<i>S. putrefaciens</i>	–	r	+	+	+	-/Ox	+	+
<i>Aeromonas</i> sp.	–	r	+	+	+	F	±	±
<i>P. phosphoreum</i>	–	Cb*	±	–	+	F	±	–

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

441 H&L: Oxidative or fermentative metabolism of glucose was performed in the medium of Hugh and
 442 Leifson (Hugh and Leifson, 1953).

443 TMAO: trimethylamine oxide (TMAO) reduction.

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456 **Table 2. Mean values of physicochemical parameters of seawater.**

Parameters	January	April	June	November
Temperature (°C)	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

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

470	Tissue	0	2	4	7	10	14	18	P	
471	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
472		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
473		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
474	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
475		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
476		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
477	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
478		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
479		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
480	<i>Pseudomonas</i> 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
481		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
482		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
483	<i>Aeromonas</i> 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
484		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
485		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
486	<i>S. putrefaciens</i>	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
487		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
488		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

489 All p-values correspond to multiple linear comparisons.

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

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

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

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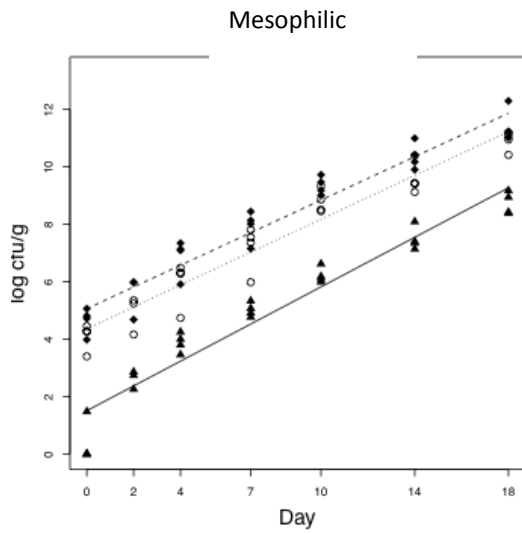
Figure 1. Log-CFU/g plotted against to observation day and its linear fitted;

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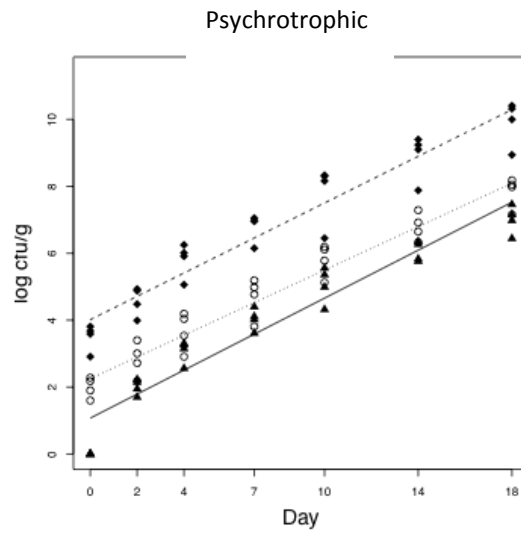
▲—— Muscle; ◆----- Gills; ○····· Skin.

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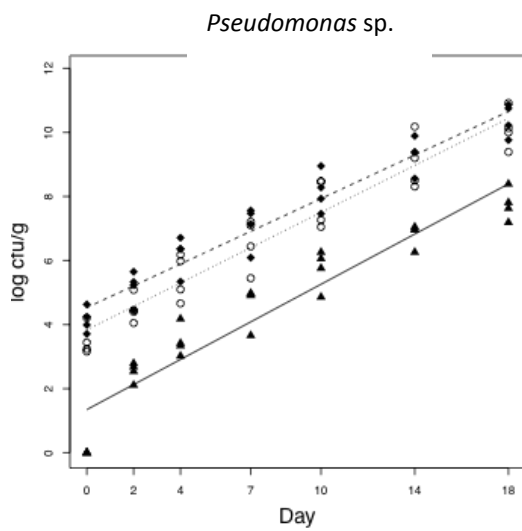
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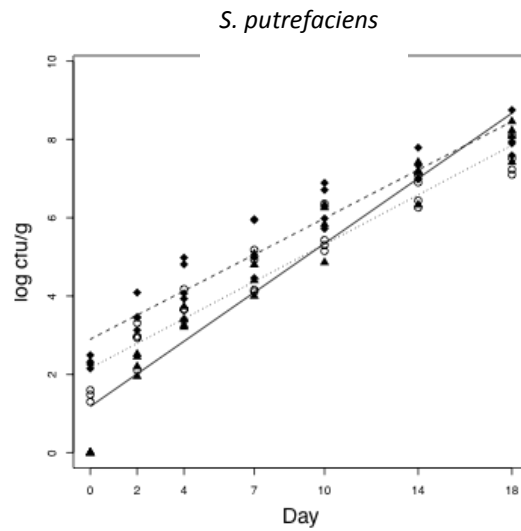
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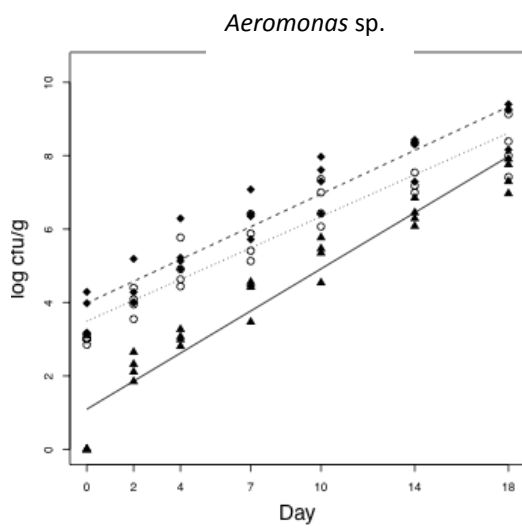
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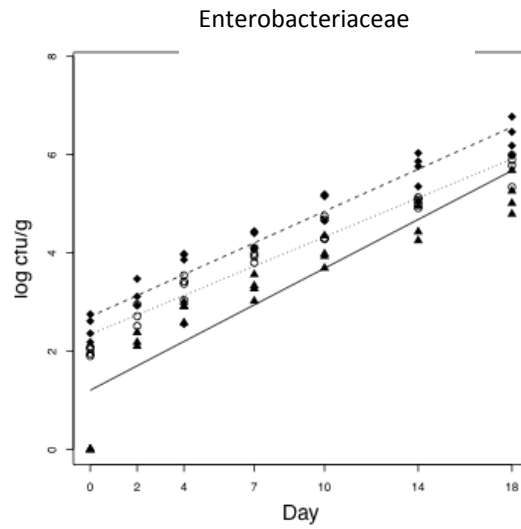
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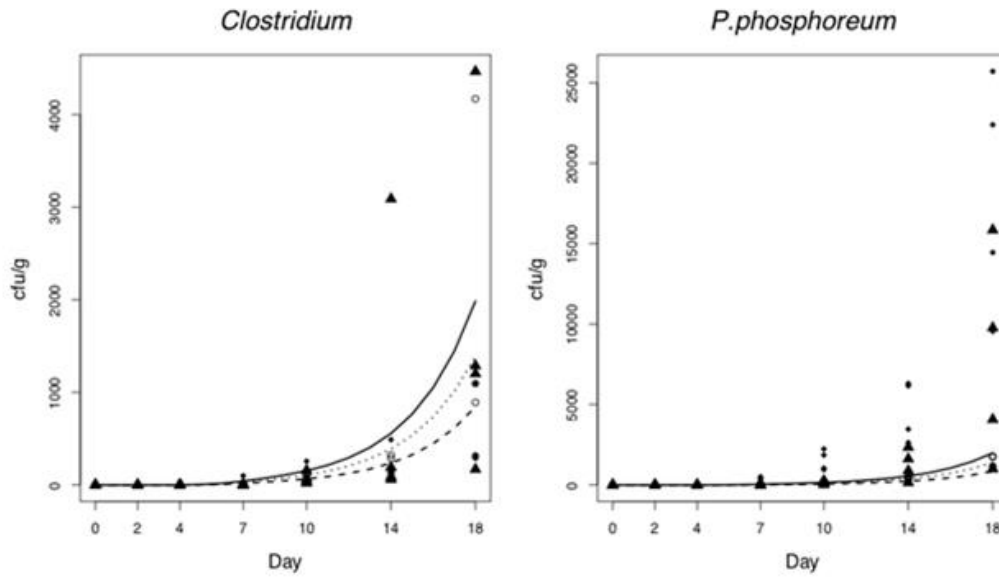


Figure 1. The cfu/g plotted against to the observation day and its fitted by means of a zero-inflated Poisson model. ▲ — Muscle; ◆ ----- Gill; ○ Skin

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