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1 **The effect of spray-dried porcine plasma on gilthead seabream (*Sparus aurata*) intestinal**
2 **microbiota**
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Abstract

The effect of spray-dried porcine plasma (SDPP) on the intestinal histological organization and autochthonous microbiota composition was evaluated in *Sparus aurata*. Fish were fed a basal diet (51% protein, 17% fat, 20.6 MJ/kg gross energy) and a diet containing 3% SDPP for 95 days (initial body weight, BW = 9.5 ± 0.2g, mean ± SD). The inclusion of SDPP promoted growth ($P < 0.05$), being fish fed the SDPP diet 6.2% (BW = 88.2 ± 1.6g) heavier than the control (BW = 82.7 ± 3.2g). SDPP increased the density of intestinal goblet cells ($P < 0.05$), whereas no differences in villi height were found ($P > 0.05$) between both groups. Intestinal microbiota was dominated by Proteobacteria (>85%) and Firmicutes (5-12%), whereas Bacteroidetes never represented more than 1.5%. γ-Proteobacteria, and Bacilli and Clostridia were the predominant classes. The short administration of SDPP (20 days) resulted in changes in microbiota diversity and richness associated to an increase in the sequences of the genus *Lactobacillus* and to a decrease in the genus *Vibrio*, whereas these changes were reverted at 95 days. Intestinal goblet cell density was not correlated to microbiota diversity and richness changes rather than to the immunostimulatory effect of the SDPP.

Keywords: goblet cell; functional feed; intestinal microbiota; seabream; spray-dried plasma.

1. Introduction

The vertebrate intestine harbours a coevolved consortium of microbes that play critical roles in the development and health of this important organ. Over the past decade, numerous studies have documented high levels of microbial diversity in vertebrate intestinal ecosystems (O'Hara & Shanahan, 2006), which is especially critical for host nutrition, immunity, health, disease prevention, development, among others. (Bäckhed,

48 2011). In the particular case of fish, the intestinal microbial population has been extensively
49 studied compared with the skin and gill microbiota (Romero, Ringø & Merrifield, 2014), and
50 its effects on digestion, metabolism, immunity and various diseases have been confirmed
51 (Nayak, 2010; Ganguly, Paul & Mukhopadhayay, 2010; Llewellyn, Boutin, Hoseinifar &
52 Derome, 2014; Montalban-Arques, De Schryver, Bossier, Gorkiewicz, Mulero, Gatlin &
53 Galindo-Villegas, 2015).

54 The intestinal microbiota of fish, as is the case of mammals, is classified as
55 autochthonous or allochthonous bacteria (see review in Ringø, Zhou, Gonzalez Vecino,
56 Wadsworth, Romero et al., 2016). The autochthonous bacteria are those able to colonize
57 the host's intestinal epithelial surface or are associated with the microvilli, while the
58 allochthonous bacteria are transient, associated with food particles or present in the
59 lumen. As Montalban-Arques et al. (2015) reviewed multiple studies have shown that by
60 virtue of their catalytic activity, the microorganisms in any vertebrate play a critical role in
61 shaping the microbiota of the intestine, its function, immune regulation, and host health.
62 Several studies using different vertebrate models like chickens, swine, mice, humans, and
63 fish have shown the possibility of applying dietary strategies to modulate the commensal
64 gastro-intestinal microbiota, which is of special importance with regard to the development
65 of "functional feeds" (Laparra & Sanz, 2010; Xu & Knight, 2015; Gonçalves & Gallardo-
66 Escárate, 2017; Dawood, Koshio & Esteban, 2018). Thus, the term "functional feeds" is
67 used to describe a particular type of food/feed that has added benefits that will improve
68 both health status and growth promoting performance of the animals, which ingest them,
69 mainly by supplying additional compounds above and beyond the basic nutritional
70 requirements for animal growth alone (Tacchi, Bickerdike, Douglas, Secombes & Martin,
71 2011). In addition to the impact of the diet on intestinal microbiota and its potential
72 beneficial effects on the organism, it is also of importance to evaluate how diet can
73 modulate intestinal microbiota as the gastrointestinal tract is one of the major ports of
74 entry for some pathogens (Romero et al., 2014; Montalban-Arques et al., 2015; Ringø et al.

75 2016). Thus, the manipulation of the host microbiota may represent a new possibility in the
76 prevention or management of pathological and physiological disorders (Pérez et al., 2010).

77 Spray-dried blood and plasma proteins are recognized as safe, high-quality feed
78 ingredients for farmed animals, including swine, cattle, poultry (Campbell, Polo, Russell &
79 Crenshaw, 2003; Ferreira, Barbosa, Tokach & Santos, 2009; Frugé, Bidner & Southern, 2009;
80 Henn, Bockor, Vieira, Ribeiro, Kessler, Albino, Rostagno, Crenshaw, Campbell & Rangel,
81 2013) and fish (Johnson & Summerfelt, 2000; Gisbert, Skalli, Campbell, Solovyev, Rodríguez,
82 Dias & Polo, 2015). Spray-dried plasma from porcine (SDPP) has an excellent amino acid
83 profile and close to 99% digestibility (Bureau, Harris & Cho, 1999) and, when included in
84 fish diets, resulted in an improvement of somatic growth and feed efficiency parameters
85 (Campbell et al., 2010; Gisbert et al., 2015). In addition, Gisbert et al. (2015) also reported
86 that SDPP was able to modulate the activity of the antioxidative defenses and increased the
87 density of goblet cells in the intestine, as well as enhanced the non-specific immune
88 response in the serum of gilthead seabream (*Sparus aurata*) juveniles. These results may be
89 partially attributed to the nutritional profile of SDPP, but also to its content in growth
90 factors, immunoglobulins and bioactive peptides (Campbell et al., 2010; Gao, Jiang, Lin,
91 Zheng, Zhou & Chen, 2011; Perez-Bosque, Polo & Torrellardona, 2016). As Dawood et al.
92 (2017) reviewed, functional feed could activate the innate immune system of aquatic
93 animals in two ways, by directly stimulating the innate immune system, or by enhancing the
94 growth of commensal microbiota as different feed ingredients and additives (e.g.
95 phytobotics, prebiotics, probiotics, immunostimulants) have been reported to modulate
96 intestinal microbiota in fish. Considering the above-mentioned SDPP properties, authors
97 decided to evaluate whether the reported positive effects of this ingredient in gilthead
98 seabream (Gisbert et al., 2015) were due to the modulation of the immune function of fish
99 and/or its microbiota. There is limited knowledge of the impact of SDP on farmed animals.
100 In pigs, the dietary inclusion of SDPP resulted in a decrease of pathogenic bacteria, as well
101 as an increase in cellulose degraders and butyric acid-producers, which were reported to

102 have positive impacts on nutrient digestion and intestinal health (Tran, Anderson, Bundy,
103 Fernando, Miller & Burkey, 2018). Thus, the objective of this study was to describe and
104 evaluate changes in microbiota richness and diversity at the genus level in *S. aurata*
105 juveniles fed 3% SDPP at 20- and 95-days post diet administration in order to better
106 characterize the effect of SDP on the organism.

107

108 **2. Material and methods**

109 *2.1 Diets*

110 A control diet (Diet C) was formulated to contain 51% crude protein, 17% crude fat and 20.6
111 MJ/kg gross energy and fulfill the nutritional requirements of juvenile gilthead seabream.
112 Based on this basal formulation, another diet named Diet SDPP was manufactured where
113 fishmeal (FM) was substituted by 3% SDPP (Appetein GS, APC Europe SL, Granollers, Spain)
114 at the expense of fishmeal LT70. Both diets were isoproteic, isolipidic and isoenergetic
115 (Table 1). Diets were manufactured by Sparos Lda (Portugal). Main ingredients were ground
116 (below 250 µm) in a micropulverizer hammer mill (Hosokawa Micron). Powder ingredients
117 and oils were then mixed according to the target formulation in a paddle mixer (RM90;
118 Mainca). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 0.8
119 and 1.5 mm) by means of a low-shear extruder (P55; Italplast). Upon extrusion, all feed
120 batches were dried in a convection oven (OP 750-UF; LTE Scientific) for 4 h at 45 °C.
121 Samples of each diet were analyzed for proximate composition analysis (Table 1).

122

123 *2.2 Fish, experimental design and general procedures*

124 Gilthead seabream fry (average body size = 9.5 g) were obtained from a commercial
125 hatchery (Piscimar, Andromeda Group, Burriana, Spain) and transported by road to IRTA-
126 Sant Carles de la Ràpita research facilities (Sant Carles de la Ràpita, Spain), where they were

127 acclimated in 2 2,000-L tanks for two weeks. After their acclimation, all fish were
128 anesthetized (tricaine methanesulfonate [MS-222], 150 mg/L) and individually weighed for
129 initial body weight (BW) and standard length (SL) to the nearest 0.1 g and 1 mm,
130 respectively, and then distributed into 8 5oo-L cyclindroconical tanks at a density of 50 fish
131 per tank (4 tanks/replicates per diet). Fish (BW = 10.6 ± 0.1 g, n = 400, mean ± standard
132 deviation, SD) were fed for 95 days with both experimental diets by means of automatic
133 feeders (ARVO-TEC T Drum 2000; Arvotec, Huutokosk, Finland) at the rate of 2.5% of the
134 stocked biomass, which approached apparent satiation. Feed ration evenly distributed in 7
135 meals per day from 8 to 18 h. Fish were regularly sampled at a monthly basis in order to
136 evaluate their growth in BW and adjust the feeding ratio.

137 During the trial, water temperature and pH (pH meter 507; Crison Instruments,
138 Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved
139 oxygen (OXI330; Crison Instruments) were 22.1 ± 0.4 °C, 7.0 ± 0.01, 36 mg/L, and 7.2 ± 0.3
140 mg/L (mean ± SD), respectively. Water flow rate in experimental tanks was maintained at
141 approximately 9.0-10.1 L/min via a recirculation system (IRTAmar™; IRTA, Barcelona, Spain)
142 that maintained adequate water quality (total ammonia and nitrite were ≤0.15 and 0.6
143 mg/L, respectively) through UV, biological, and mechanical filtration. Photoperiod followed
144 natural changes according to the season of the year (November to February; 40°37’41” N).

145 After 20 and 95 days of diet administration, fish were anaesthetized as previously
146 described and their BW and SL measured as indicated. Before sampling, fish were fasted for
147 18 h. Six fish per tank were sacrificed with an overdose of the same anesthetic and their
148 whole intestine were aseptically removed with a scalpel. A small piece of the anterior
149 intestine (0.5 cm) was fixed in buffered formaldehyde (pH 7.2) and the rest of the intestine
150 stored separately at -20 ºC until further microbiological analysis.

151 All animal experimental procedures were conducted in compliance with the
152 experimental research protocol approved by the Committee of Ethics and Animal

153 Experimentation of the Institut de Recerca i Tecnologia Agroalimentàries and in accordance
154 with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory
155 animals.

156

157 *2.3 Histological analysis*

158 Fixed anterior-mid intestine sections were dehydrated in a graded series of ethanol, cleared
159 with xylene, embedded in paraffin, and cut in serial sections (3 µm thick). Transverse
160 sections were stained with hematoxylin-eosin, observed with a light microscope (Leica DM
161 LB; Leica Microsystems, Wetzlar, Germany) and photographed (Olympus DP70 Digital
162 Camera; Olympus Imaging Europa GmbH, Hamburg, Germany). Digital images (600 dpi)
163 were processed and analyzed using an image analysis software package (ANALYSIS; Soft
164 Imaging Systems GmbH, Münster, Germany). Measurements of total goblet cell number
165 (full and empty) and villi height were based on the analysis of 8 to 10 randomly chosen
166 fields from the intestinal mucosa of 20 fish per dietary group (Gisbert, Castillo, Skalli,
167 Andree & Badiola, 2013). Goblet cell counts in intestinal villi were expressed over a contour
168 length of 100 µm, whereas villi height was measured as indicated in Figure 1.

169

170 *2.4 Microbiota analysis*

171 Individual digestive tracts of gilthead seabream were washed several times in sterile PBS
172 (pH 7.2); the mucus was scraped off with a sterilized scalpel and collected in sterile 1.5-mL
173 tubes. Samples were centrifuged at 1,000 *g* for 5 min. Four samples from the same
174 experimental group, one from each tank, were pooled and total DNA was extracted from
175 each pool (Hao, Wu, Li, Yu, Wang & Ling, 2017). The samples were mixed with 300 mL of re-
176 suspension buffer (0.1 M Tris-HCl, 0.01 M NaCl, 0.1 M EDTA, pH 8) and 300 mL of lysis
177 buffer (0.1 M Tris-HCl, 0.1 M EDTA, 0.01 M NaCl, 1% SDS, pH 8.0), gently inverting the tube

178 to mix thoroughly. Afterwards, the samples were treated with 32 mL NaCl 6 M and
179 proteinase K (150 mg/mL) at 55 ºC for 2 h. Next, we followed with RNAase A treatment (10
180 mg/mL) at 37 ºC for 1 h. Next, 6 M NaCl was added to reach a final concentration of 1.5 M.
181 The solution was chilled on ice for 10 min followed by a new centrifugation (21,000 x *g*, 3
182 min). The clear supernatant containing genomic DNA was transferred to another tube
183 containing an equal volume of isopropanol. The tubes were inverted gently several times.
184 The DNA was pelleted by centrifugation (21,000 x *g*, 3 min). The DNA pellet was then
185 washed in 70% ethanol, and the dried DNA pellet was resuspended in 100 mL of TE buffer
186 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4 ºC (Tapia-Paniagua, Chabrillón, Díaz-
187 Rosales, de la Banda, Lobo, Balebona & Moriñigo, 2010).

188 DNA samples of fish receiving the same Diet (C and SDPP) and sampled at same time (20
189 and 95 days) were pooled to simplify the microbe analysis using next generation
190 sequencing technique and subsequent microbiota analysis. Each pool represented a
191 particular digestive of each treatment and time sampled and this strategy was chosen in
192 order to reduce sample dispersion (Hao et al., 2017). Libraries were constructed by
193 Chunlab, Inc., (Seoul, South Korea) using the Illumina MiSeq Platform. The primers used
194 were 341F CCTACGGGNGGCWGCAG and 805R ACTACHVGGGTATCTAATCC (ChunLab),
195 targeting V3-V4 regions of 16 S rRNA gene. . Sequences were analyzed using
196 CLcommunity™ software (ChunLab). Sequences of a length less than 200 nt were excluded
197 from the analysis. The data were filtered for noisy sequences, checked for the presence of
198 chimeras, and binned into OTUs (Peiffer et al., 2013) at the 97% sequence similarity were
199 compared using the Greengene database. Rarefaction curves were obtained by plotting the
200 number of observed OTUs against the number of sequences. The number of sequences in
201 all samples was normalized at 60,000. A representative sequence of each OTU was
202 taxonomically classified. In addition, Shannon-Wiener and Chao1 indexes were calculated
203 to determine the diversity and richness, respectively. To analyze the distribution of OTUs at
204 phylum, class, family and genus level with relative abundances >1% of the total reads across

205 all samples, a heatmap was also constructed using ascendant clustering based on Euclidian
206 distances. The data matrix's rows and columns were then permuted according to
207 corresponding clusterings, which brought similar columns closer to each other and similar
208 lines closer to each other. The heatmap reflected the data in the permuted matrix (data
209 values were replaced by corresponding color intensities) using XLSTAT software version
210 2019 (Addinsoft, Spain).

211

212 *2.5 Statistical analysis*

213 The mean values of BW and SL were expressed as mean ± standard deviation (SD). The
214 calculation was based on individual BW and SL values of all the fish belonging to the same
215 treatment, and consequently, the SD describes the dispersion of the individual values. The
216 mean values of goblet cell density and villi height were expressed as mean ± standard error
217 of the mean (SEM). These two histological parameters were calculated using the values of
218 the replicates (n = 4 for each treatment), and the SEM quantifies the error in calculating the
219 mean of the population from the tank values. Mean values were compared by means of a t-
220 test, and the level of significance set at $P < 0.05$.

221

222 **3. Results**

223 Results in terms of somatic growth of *S. aurata* juveniles fed the experimental diets are
224 shown in Table 2. At 20 days after diet administration, both experimental groups were
225 similar in terms of BW and SL (P > 0.05), whereas at the end of the study fish fed the SDPP
226 diet were 6.2% heavier than those of the control diet (P < 0.05). No differences in SL were
227 observed at the end of the trial (P > 0.05).

228 Histological sections revealed that the organization of the intestinal mucosa in
229 seabream fed both diets was normal (Figure 1). The inclusion of the SDPP in feeds for

230 seabream did not affect the length of intestinal villi at either of both sampling times, but it
231 significantly increased the density of intestinal goblet cells ($P < 0.05$). Fish fed the SDPP diet
232 showed a higher number of goblet cells than the control group at both sampling dates
233 (Table 3).

234 The inclusion of SDPP in the diet had a higher effect on the autochthonous
235 intestinal microbiota at 20 days (diet SDPP-20d) after feeding when compared to the group
236 C-20d. In particular, SDPP resulted in a reduction of the richness of the microbial
237 community (Chao 1 index = 498.56) that was in accordance with a lower number of families
238 and genera detected found in this group (170 and 344, respectively) (Table 4). On the
239 contrary, the value of Shannon-Wiener index at 20 days was not very different to the value
240 calculated for the control group (3.17 *vs.* 2.77). However, the above-mentioned effect on
241 microbiota richness and evenness was not observed after 95 days of feed administration
242 (diet SDPP-95d), since samples from this experimental group showed similar values of
243 Chao1 and Shannon-Wiener indexes to those calculated for the C-95d group (547.64 *vs*.
244 507.51 and 2.71 *vs.* 2.76, respectively) (Table 4).

245 Regarding microbiota intestinal composition in terms of bacteria phylum, the most
246 abundant phylum detected in all samples was Proteobacteria, representing more than 85%
247 of all sequences analyzed, whereas γ-Proteobacteria was the most abundant class (>73%) of
248 Proteobacteria (Figure 2A). Other predominant phyla in all samples were Firmicutes
249 (abundance values ranging from 4.66% to 8.45%) and Bacteroidetes (abundance values
250 slightly higher than 1%). In agreement with previous results, microbiota analysis at a family
251 taxonomic level showed a very homogeneous composition in all samples analyzed with a
252 clear dominance of the bacteria families included in γ-*Proteobacteria* , the dominant class in
253 all cases (Figure 1B), such as *Pseudomonadaceae* and *Xanthomonadaceae* (abundance
254 values ranging from 29.28 to 34.19%) (Figure 3A), followed by *Enterobacteriaceae*
255 (abundance values ranging from 10.0 to 13.0%), whereas families included in β–

256 *Proteobacteriae*, such as *Alcaligenaceae* and *Comamonadaceae* showed lower abundance
257 values than 5%. Others, families such as *Sphingomonadaceae* (α–*Proteobacteriae*) and
258 *Ruminococcaceae* (Firmicutes) were also detected in all samples, but in abundance levels
259 slightly higher than 1%. *Vibrionaceae* and *Ruminococcaceae* reached lower abundance
260 levels ranging from 1.5% to 2.0%. In addition, *Lactobacillaceae* was a predominant family in
261 the intestines of gilthead seabream samples from C-95d (4.6%), as well as in fish fed the
262 diet SDPP at 20 days after administration (2.1%), whereas in SDPP-95d samples their
263 abundance decreased.

264 At a genus taxonomic level, the dominant genera in all samples were *Pseudomonas*
265 (*Pseudomonadaceae*), *Stenotrophomonas* (*Xanthomonadaceae*) and *Enterobacter*
266 (*Enterobacteriaceae*) including more than 65% of total sequences analyzed (Figure 3B).
267 Other genera such as *Achomobacte*r (*Alcaligenaceae*), *Luteibacter* (*Xanthomonadaceae*),
268 *Pelomonas* (*Comamonadaceae*) and *Sphingomonas* (*Sphingomonadaceae*) were in all
269 samples but in lower levels. Differences regards to the abundance of genera such as
270 *Bradyrhizobium, Vibrio* and *Lactobacillus* were observed between diets control and SDPP at
271 20 days, whereas the abundance of genus *Lactobacillus* showed a presence higher than 2%
272 only in seabream fed the control diet at 95 days (4.57%).

273

274 **4. Discussion**

275 Present results confirmed that the inclusion of SDPP in seabream diets promoted somatic
276 growth, as it was previously shown in this species by Gisbert et al. (2015) and in rainbow
277 trout (*Oncorhynchus mykiss*) (Johnson & Summerfelt, 2000). These results are mainly
278 attributed to the presence of diverse functional proteins like growth factors, cytokines and
279 other biologically active compounds that may contribute to its positive effects on animal
280 performance), as well as in an improvement of diet digestibility (Hou, Wu, Dai, Wang &
281 Wu, 2017). However, the main findings of this study were related to the impact of this

282 ingredient on intestinal microbiota diversity and composition, a sort of information that is
283 absent in fish.

284 Diets containing SDPP have demonstrated to exert beneficial effects of SDPP on the
285 intestine in relation to the mucosal permeability, epithelial defensing secretion and anti-
286 inflammatory effects (Pérez-Bosque, Polo, Russell, Campbell, Weaver& Moretó, 2010;
287 Gisbert et al., 2015; Pérez-Bosque et al., 2016). However, there is very limited information
288 about the effect of this supplement on the intestinal microbiota (Tran et al., 2018), and
289 whether the above-mentioned positive effects are due to changes in the intestinal
290 microbiota, bioactive compounds in SDPP or both. In our study, SDPP showed limited
291 effects on the intestinal microbiota in gilthead seabream, although alpha-diversity indexes
292 such as Chao1 showed differences in the SDPP group at 20 days. Intestinal microbiota
293 richness in fish fed the SDPP diet was reduced compared to the control diet. However, this
294 decrease did not imply a higher dominance of certain microbial groups, because Shannon-
295 Wiener index indicated that the evenness in both groups corresponded to values of
296 environments with a moderate diversity.

297 Although microbiota composition seems to differ among fish species, it is generally
298 dominated mainly by the phyla Proteobacteria, Firmicutes and Actinobacteria (Sullam,
299 Newman, Silverman, Turner & Lilley, 2012; Ringø et al., 2016). In the current study,
300 Proteobacteria (>85% of the analyzed sequences) and Firmicutes (5-12%) were the most
301 abundant phyla in the autochthonous intestinal microbiota of gilthead seabream. These
302 results were in agreement to those reported by different authors in the same species
303 (Cordero, Guardiola, Tapia-Paniagua, Cuesta, Meseguer, Balebona, Moriñigo & Esteban, M.,
304 2015; Estruch, Collado, Peñaranda, Tomás Vidal, Jover Cerdá, Pérez Martínez, & Martinez-
305 Llorens, 2015; Piazzon, Calduch-Giner, Fouz, Estensoro, Simó-Mirabet, Puyalto, Karalazos,
306 Palenzuela, Sitjà-Bobadilla & Pérez-Sánchez, 2017), as well in other temperate marine fish
307 species (Cardá-Dieguez, Mira & Foutz, 2014; Tapia-Paniagua, Vidal, Lobo, de la Banda,

308 Esteban, Balebona & Moriñigo, 2015) and salmonids (Merrifield, Burnard, Bradley, Davies
309 & Baker, 2009; Dehler, Secombes & Martin, 2017). In our study, this phylum never
310 represented more than 1.5% of the sequences analyzed, whereas other 22 phyla were
311 found, but these were <1% of the total sequences. Actinobacteria have also been reported
312 as a predominant phylum in gilthead seabream (Estruch et al., 2015) ranging 25% to 40%.
313 However, under current experimental conditions and similarly to Piazzon et al. (2017),
314 Actinobacteria never reached similar levels, differences that might be attributed to
315 different physiological, environmental and dietary conditions of examined fish (Ringø et al.,
316 2016). These results may be also due to the nature of the samples (intestinal content *vs.*
317 mucus) and the protocols used for obtaining and their storage (Piazzon et al., 2017).

318 γ-Proteobacteria was the predominant class detected in our study, results that
319 were in concordance with other studies conducted in this species (Tapia-Paniagua et al.,
320 2011; Cordero et al., 2015; Estruch et al., 2015; Piazzon et al., 2017), as well as in
321 Senegalese sole (Tapia-Paniagua et al., 2015). Kormas, Meziti, Mente & Frentzos (2014)
322 reported that β-Proteobacteria were the most dominant class of autochthonous intestinal
323 microbiota in gilthead seabream. In our study, α- and β-Proteobacteria also were
324 predominant groups, but their frequencies were never higher than γ-Proteobacteria. It is
325 well known that different fish rearing conditions (environmental and dietary factors) have a
326 significant influence on the intestinal microbiota (Llewellyn, Boutin, Hoseinifar & Derome,
327 2014; Kormas et al., 2014; Dehler et al., 2017; Kononova, Zinchenko, Muranova, Belova &
328 Miroshniko, 2019), which may explain the above-mentioned differences. Regarding the
329 Firmicutes, the predominant classes were Bacilli and Clostridia, results in agreement with
330 those reported by Estruch et al. (2015), who observed that members of these classes were
331 predominant in gilthead seabream.

332 At the genus level, *Pseudomonas*, *Enterobacter* and *Stenotrophomonas* were the
333 most predominant genera, while *Achromobacter* and *Luteibacter* were less abundant.

334 *Enterobacter*, *Achromobacter* and *Pseudomonas* have been described as some of the most
335 dominant genera in marine fish (Tapia-Paniagua et al., 2015; Parlapani & Boziaris, 2016;
336 Wang, Zhang, Li & Lin, 2016). Strains of these genera have shown to have positive effects
337 on fish host due to their ability to degrade cellulose and even some *Pseudomonas* and
338 *Enterobacter* strains have been proposed as probiotic bacteria (Nayak, 2010).
339 *Achromobacter* has also been reported in farmed Atlantic cod (*Gadus morhua*) (Ringø,
340 Spersta, Myklebus, Refstie & Krogdahl, 2006), as well as in wild jack mackerel (*Trachurus*
341 *japonicus*). This genus has been reported to hydrolysable tannin by the enzyme tannase
342 (Lewis & Starkey, 1969). Harboring intestinal microflora having the ability to degrade
343 tannins to innocuous compounds might be viewed as a strategy for overcoming adverse
344 effects of tannins. Thus, it would be logical to assume that the presence of tannin-
345 degrading microbiota in the intestine of gilthead seabream, an omnivorous species, could
346 be the result of the inclusion of feed ingredients derived from vegetal feedstuffs, as it has
347 been described in herbivorous and omnivorous fishes (Mandal & Ghosh, 2013).
348 *Stenotrophomonas* genus has been found in the microbiota of several farmed fish species
349 like seabream, Atlantic salmon (Navarrete, Espejo & Romero, 2009) ad rainbow trout
350 (Heikkinen et al. 2006), although it is typically described as a predominant soil bacterial
351 group (Soltani, Zaheri-Shoja, Hamze, Hosseyni-Moghaddam & Pakvaz, 2016) and plants
352 (Gandolfi, Canedoli, Imperato, Tagliaferri, Gkorezis, Vangronsveld, Schioppa, Papacchini &
353 Franzetti, 2017). These last results are interesting because this genus was found in rainbow
354 trout fed a soybean meal based diet and it was absent in fish fed a diet based on fishmeal
355 (Heikkinen, Vielma, Kemiläinen, Tiirola, Eskelinen, Kiuru, Navia-Paldanius & von Wright,
356 2006). On the other hand, *Luteibacter* is a genus has also been isolated mainly from soils,
357 although this genus is rare in fish and crustaceans microbiota (Tao, Du, Wang, Dong, Yu,
358 Ren, Sima & Xu, 2018). In our study, its abundance was about 5% in all samples. Some
359 strains have showed ability to produce lipases (Bresciani, Santi, Macedo, Abraham,

360 Vainstein & Beys-da-Silva, 2014), glycosidases (Fu, Yin, Wu & Yin, 2014) and to degrade and
361 convert different xenobiotic pollutants and organic compounds (Cui, Wu, Zhao & Yin, 2016).

362 The above-mentioned differences in microbiota found at day 20 were associated to an
363 increase in the level of sequences related to the family Lactobacillaceae and the
364 *Lactobacillus* genus. *Lactobacillus* is commonly found in different freshwater and marine
365 species, including gilthead seabream (Estruch et al., 2015), and their abundance is
366 commonly modulated by different dietary elements (e.g. vitamins, protein sources, feed
367 additives, etc.) (Ringø et al., 2016). In this sense, several authors have reported the
368 beneficial effects of lactic acid bacteria include the promotion of somatic growth,
369 improvement of feed efficiency parameters and prevention of intestinal disorders and pre-
370 digestion of anti-nutritional factors present in the ingredients (Gatesoupe, 2010). Present
371 results found in seabream fed the SDPP diet were in agreement with those studies
372 reporting that pig diets containing SDP administered during nursery and weanling periods
373 promoted the growth of lactobacilli in the intestine (Torrallardona. Conde, Badiola, Polo &
374 Brufau, 2003; Tran, Anderson, Bundy, Fernando, Miller & Burkey, 2018). However, in our
375 study the abundance of *Lactobacillus* was dependent of the time, because at the end of the
376 trial at 95 days, the level of Lactobacillae family and *Lactobacillus* genus were <1% in
377 seabream fed the SDPP diet, whereas in specimens fed the control diet the level was higher
378 (4.5%). The above-mentioned differences between both groups sampled at two different
379 times could be due to the stage fish development as its well-known that the host's age and
380 weight are factors affecting the composition of the intestinal microbiota (Li, Long,
381 Gatesoupe, Zhang, Li, A., Gong, 2015; Stephens, Burns, Stagaman, Wong, Rawls, Guillemin
382 & Bohannan, 2016). In addition, the morphology of the intestine is also a host factor
383 affecting the intestinal microbiota (Wang, Ran, Ringø & Zhou, 2017). Several studies carried
384 out with chickens and piglets reported that the dietary administration of *Lactobacillus*
385 strains (Forte, Manuali, Abbate, Papa, Vieceli, Tentellini, Trabalza-Marinucc &, Moscati,
386 2018) increased the level of *Lactobacillus* in the intestine, which was associated with

387 increases of the villi height. In contrast with these results, in our study fish fed the diet
388 SDPP after 95 days showed the highest villi heigth, but the level of *Lactobacillus* was lower
389 than 1%, whereas the villi height in fish receiving the control diet was not significant
390 different but they showed a higher level of *Lactobacillus*.

391 The mucus layer produced by intestinal goblet cells that is located at the interface between
392 the intestinal epithelium and the microbiota is considered a key factor in the crosstalk
393 between the intestinal epithelium and the microbiota. -Studies with gnotobiotic model
394 organisms have reported that the number and function of goblet cells were modulated by
395 the microbiota (Kandori, Hirayama, Takeda & Do, 1996). Our results were not in agreement
396 with the former hypothesis since the higher density of intestinal goblet cells in fish fed the
397 SDPP diet were not correlated to changes in microbiota composition; thus, future studies
398 will be necessary to check this aspect and the ability of human *Lactobacillus* species to
399 adhere to mucus (Chabrillón, Ouwehand, Díaz-Rosales, Arijo, Martínez-Manzanares,
400 Balebona & Moriñigo, 2006). Thus, our results indicated that such differences in goblet cell
401 density between SDPP and control groups may be mostly due to an enhancement of the
402 intestinal innate immune function in fish fed SDPP (Gisbert et al., 2015) rather than changes
403 in microbiota.

404 In conclusion, present results showed that the inclusion of SDPP in diets for seabream
405 promoted their somatic growth, increased the density of intestinal goblet cells and did not
406 result in major changes in autochthonous microbiota although some differences, such as
407 reduction of richness, were detected after the short administration of SDPP. The increase in
408 intestinal goblet cell density was not correlated to changes in microbiota diversity and
409 richness rather than to the immunostimulatory effect of SDPP. These results are of practical
410 relevance since they proved that SDPP is a safe ingredient for aquafeeds.

411

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416 histological samples.

417

418 **Data availability statement**

419 The data that support the findings presented in this study are available in "Repositorio
420 IRTA" at http://repositori.irta.cat/

421

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634

635 Figure 1. Histological images of the intestine in gilthead seabream *(Sparus aurata*) fed for 95
636 days with control (C) and 3% of porcine spray-dried plasma (SDPP) diets. A. Transversal
637 section of the mid-anterior intestine of a fish fed the C diet showing how the villi height were
638 measured between the *lamina propia* and the tip of the villi from several villi (with arrows).
639 B. Transversal section of the mid-anterior intestine of a fish fed the SDPP diet. C, D. General
640 view of longitudinal sections of the mid-anterior intestine of fish fed the C and SDPP diets,
641 respectively. Goblet cell counts in intestinal villi were expressed over a contour length of 100
642 µm from of 5-8 randomly chosen fields. Note the higher abundance of goblet cells in the
643 section from fish fed the SDPP diet. E, F. Detail of intestinal villi of fish fed the C and SDPP
644 diets, respectively. *Abbreviations*: GC, goblet cell; L, lymphocyte; LP, *lamina propia*; MV,
645 microvilli; PC; plasmatic cell; TM, *tunica muscularis*; V, villi. *Staining:* hematoxylin-eosin.
646
647
648 Figure 2. Heat map showing the number of sequences detected of each treatment (expressed
649 as log scale) from the intestinal microbiota of gilthead seabream *(Sparus aurata*) from lower
650 (red) to higher (green) abundance. Rows indicate Filo (A) and Class (B) OTUs and columns
651 indicate the gut juveniles fish fed for 20 and 95 days with the control diet (C-20 days and C-
652 95 days), and a diet containing 3% of porcine spray-dried plasma (SDPP) (SDPP-20 days and
653 SDPP-95 days).
654
655 Figure 3. Heat map showing the number of sequences detected of each treatment (expressed
656 as log scale) from the intestinal microbiota of gilthead seabream *(Sparus aurata*) from lower
657 (red) to higher (green) abundance. Rows indicate Family (A) and Genera (B) OTUs and
658 columns indicate the gut juveniles fish fed for 20 and 95 days with the control diet (C-20 days
659 and C-95 days), and a diet containing 3% of porcine spray-dried plasma (SDPP) (SDPP-20 days
660 and SDPP-95 days).
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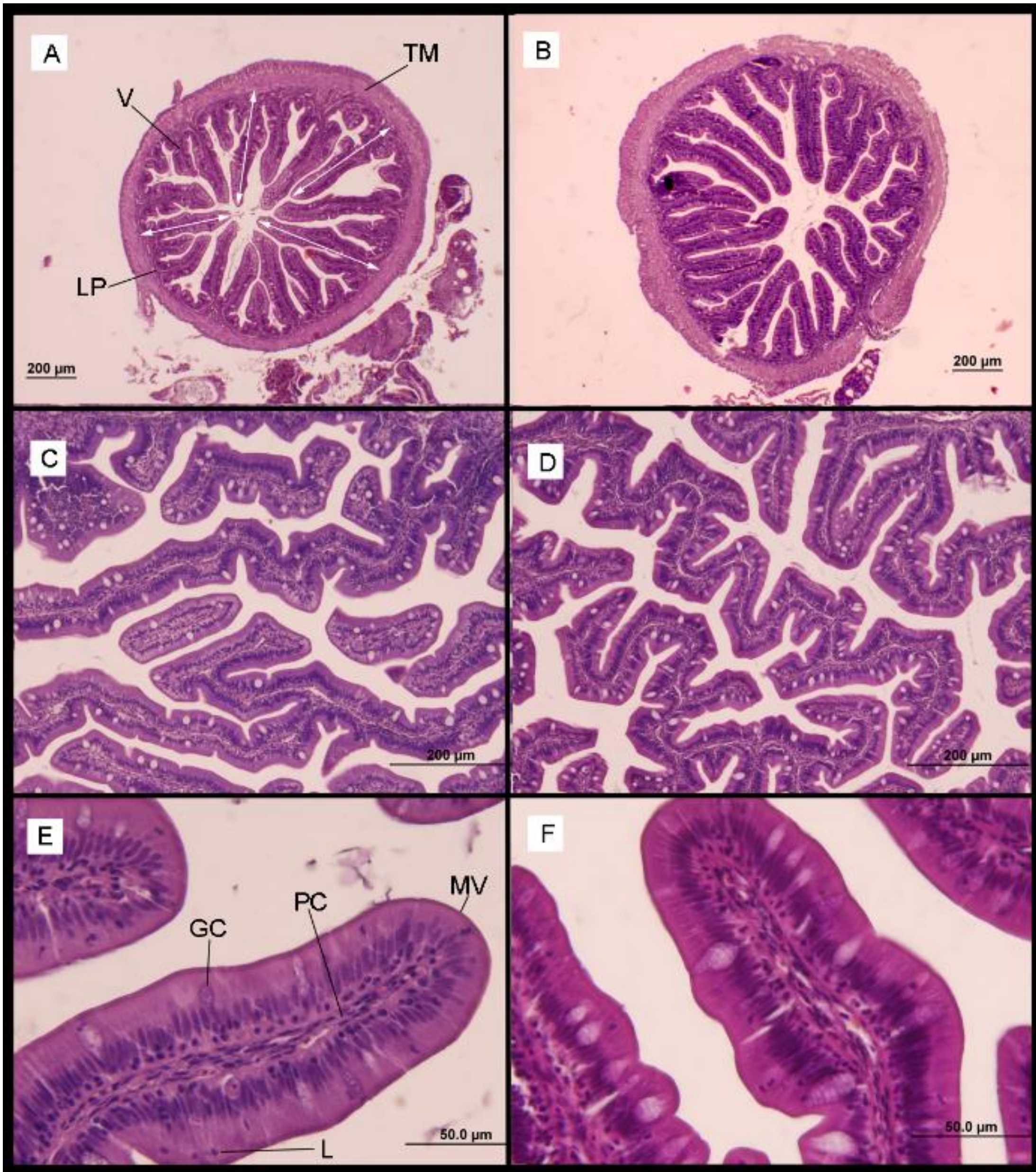

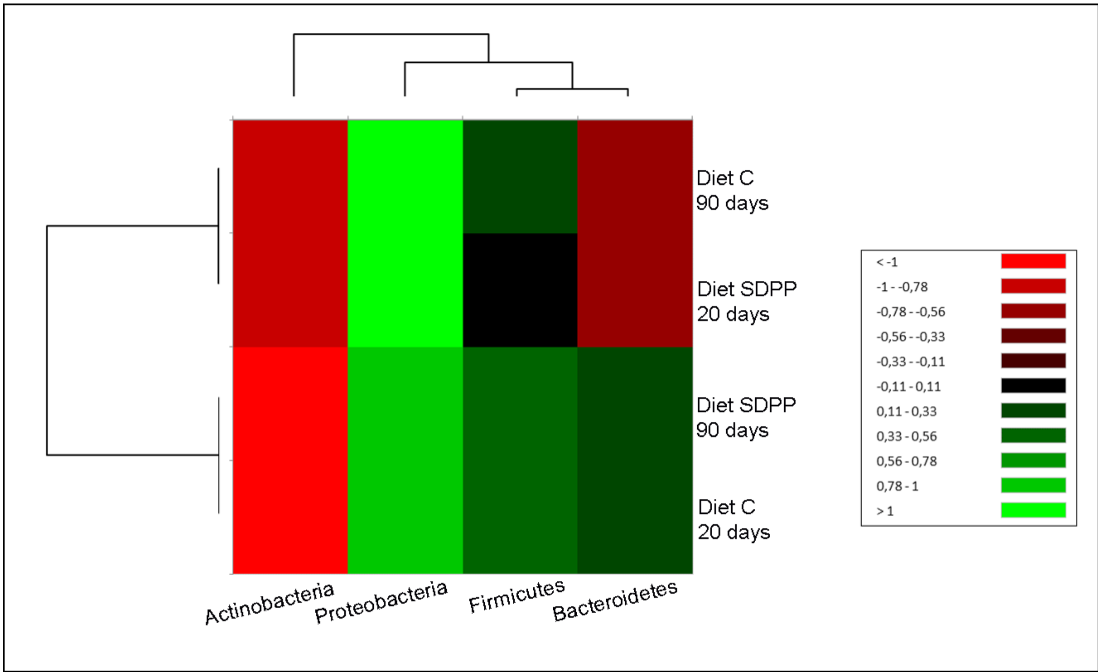

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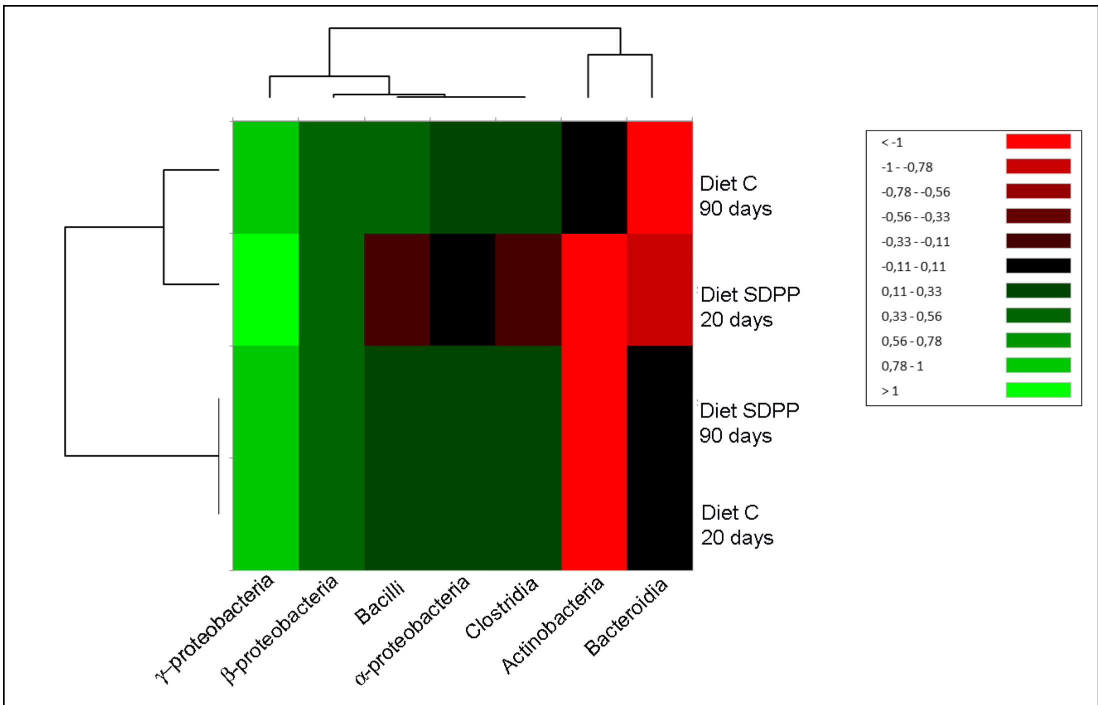

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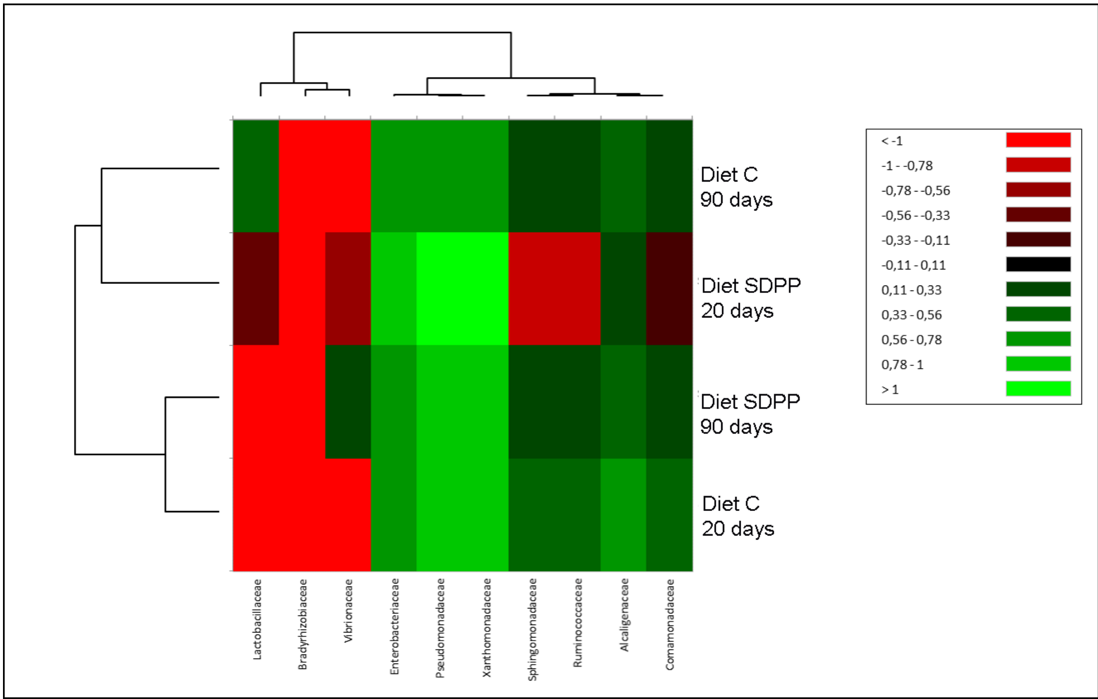

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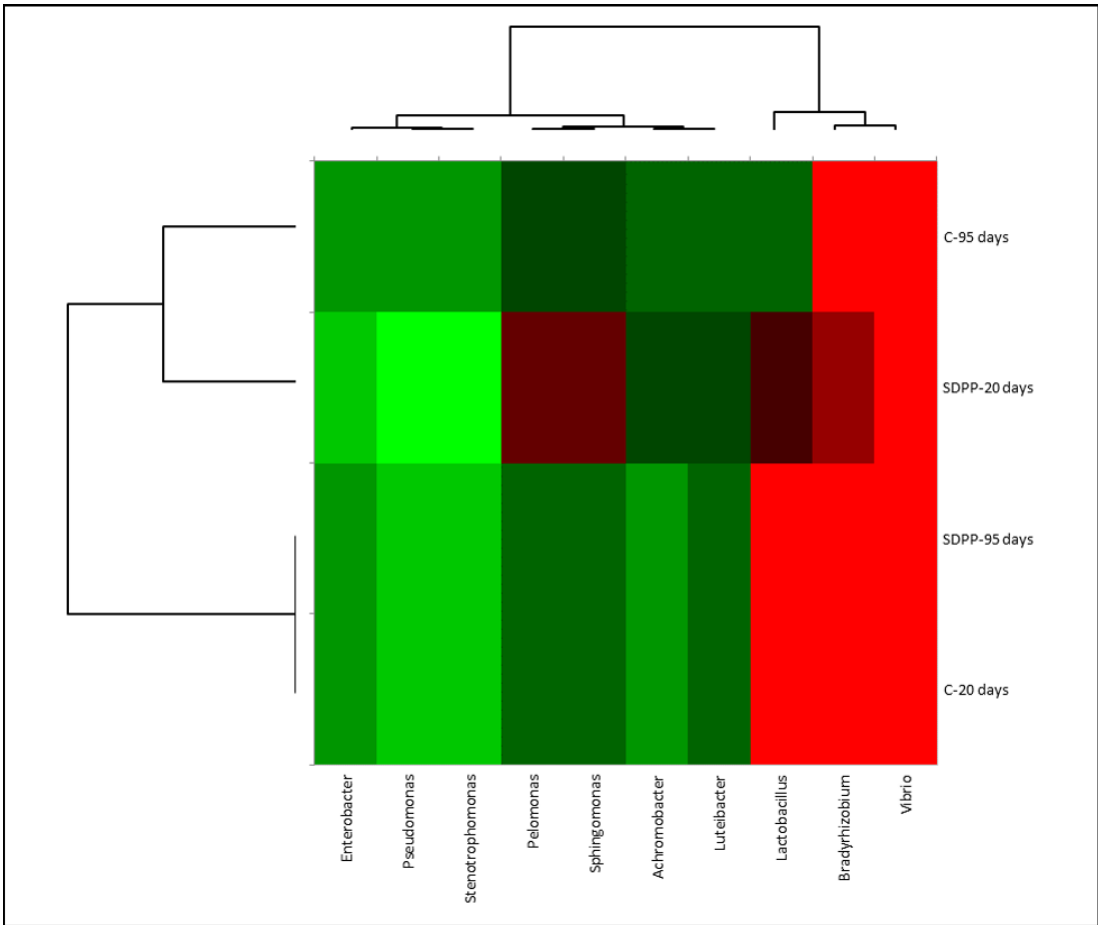

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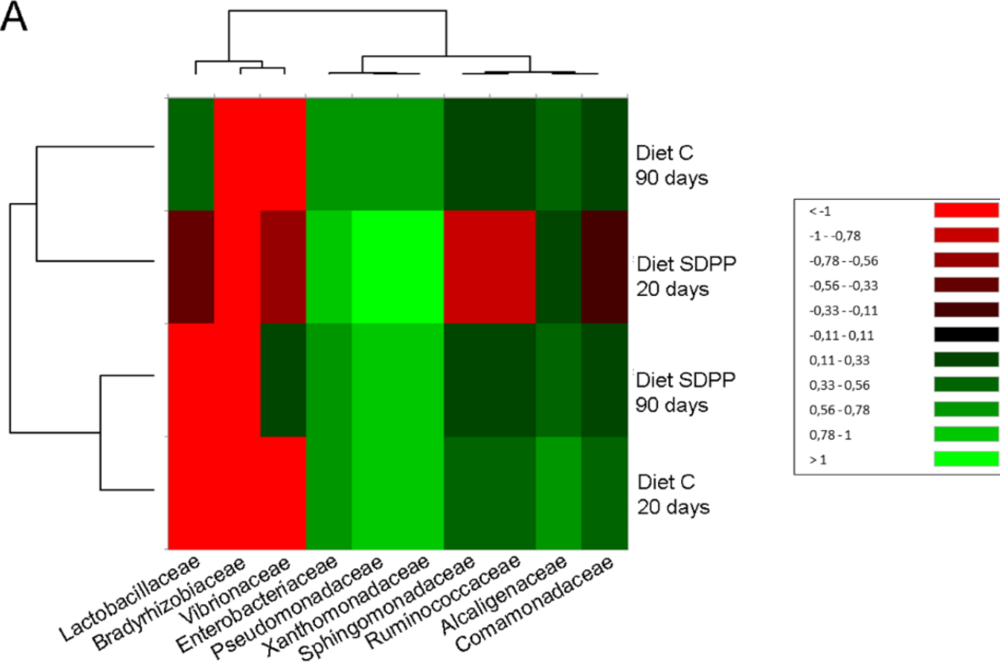

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675 Table 1. Ingredient list and proximal composition of experimental diets used in the current

676 study.

Ingredients, g/kg	Diet C	Diet SDPP
Fishmeal LT 70	369.0	333.0
Fishmeal 60	125.0	125.0
CPSP 90	40.0	40.0
Squid meal	60.0	60.0
Appetein H520522 (APC)	-	30.0
Wheat Gluten	76.0	76.0
Soybean meal 48 (micronized)	70.0	70.0
Wheat meal	77.0	77.0
Pea starch	45.0	48.0
Fish oil	112.0	114.5
Vit & Min Premix PV01	10.0	10.0
Choline chloride	1.0	1.0
Soy lecithin	5.0	5.0
Binder (guar gum)	10.0	10.0
Proximate composition, %		
Crude protein	51.1 ± 0.06	51.2 ± 0.04
Crude fat	17.2 ± 0.10	17.1 ± 0.08
Ash	11.8 ± 0.2	11.7 ± 0.1
Gross energy (MJ/kg)	20.56	20.69

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679

680 Table 2. Somatic growth in terms of body weight (BW, g) and standard length (SL, cm) of
681 gilthead seabream (*Sparus aurata*) juveniles fed a control and a diet containing 3% of
682 porcine spray-dried plasma (SDPP) at two different times after feed administration.
683 Presented values are mean ± SD. Different letters within the same row indicate the
684 existence of statistically significant differences between both experimental groups (t-test, P
685 = 0.019).

	Diet C		**Diet SDPP**	
	BW (g)	SL (mm)	BW (g)	SL (mm)
Initial	10.6 ± 0.1	7.7 ± 0.2	10.5 ± 0.1	7.6 ± 0.2
20 days	23.1 ± 0.6	9.5 ± 0.1	22.9 ± 0.7	9.4 ± 0.1
95 days	82.7 ± 3.2 b	14.6 ± 0.2	88.2 ± 1.6 a	14.8 ± 0.1

686

687

688 Table 3. Villi height (µm) and goblet cell density (number of cells in 100 µm of epithelium) in
689 the anterior-mid intestine of gilthead seabream (*Sparus aurata*) juveniles fed a control and
690 a diet containing 3% of porcine spray-dried plasma (SDPP) at two different times after feed
691 administration. Presented values are mean ± SEM. Different letters within the same row
692 indicate the existence of statistically significant differences between both experimental
693 groups (t-test, P < 0.05).

	Diet C		**Diet SDPP**	
	Villi height	Goblet cell density	Villi height	Goblet cell density
20 days	568.5 ± 111.2	0.79 ± 0.04 b	622.4 ± 98.4	0.91 ± 0.03 a
95 days	1,003 ± 376	0.83 ± 0.06 b	1,251 ± 289	1.09 ± 0.09 a

694

695

696 Table 4. Number of reads, diversity indexes and assigned taxa of authochtonus intestinal
697 microbiota samples from gilthead seabream (*Sparus aurata*) juveniles fed a control and a
698 diet containing 3% of porcine spray-dried plasma (SDPP) at two different times after feed
699 administration.

	Diet / administration time			
	C – 20 days	SDPP – 20 days	C – 95 days	SDPP – 95 days
Number of reads	70,183	118,403	88,842	91,277
Chao 1 index	746.80	498.56	507.51	547.64
Shannon-Wiener index	2.77	3.17	2.76	2.71
Number of families	218	170	166	189
Number of genera	451	344	405	378

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Figure captions

711

712 Figure 1. Histological organization of the intestine in gilthead seabream (*Sparus aurata*)
713 juveniles fed a diet containing 3% of porcine spray-dried plasma (SDPP). Staining:
714 hematoxylin-eosin.

715

716 Figure 2. Comparison at level of phylum (a) and class (b) of the autochthonous intestinal
717 microbiota composition of gilthead seabream (*Sparus aurata*) juveniles fed for 20 and 95
718 days with the control diet (C-20 days and C-95 days) and a diet containing 3% of porcine
719 spray-dried plasma (SDPP) (SDPP-20 days and SDPP-95 days).

720

721 Figure 3. Comparison at level of family (a) and genus (b) of the autochthonous intestinal
722 microbiota composition of gilthead seabream (*Sparus aurata*) juveniles fed for 20 and 95
723 days with the control diet (C-20 days and C-95 days) and a diet containing 3% of porcine
724 spray-dried plasma (SDPP) (SDPP-20 days and SDPP-95 days).

725

726

727 Supplementary file 1. Rarefaction curves obtained from the analysis of the composition of
728 the intestinal microbiota of gilthead seabream (*Sparus aurata*) fed for 20 and 95 days with
729 Control diet (control) (C-20 days) and a diet containing 3% of porcine spray-dried plasma
730 (SDPP) (SDPP-20 days and SDPP-95 days).