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Effects of calcium carbonate inclusion in low fishmeal diets on growth, gastrointestinal pH, digestive enzyme activity and gut bacterial community of European sea bass (*Dicentrarchus labrax* L.) juveniles

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26 **Effects of calcium carbonate inclusion in low fishmeal diets on growth,**
27 **gastrointestinal pH, digestive enzyme activity and gut bacterial community of**
28 **European sea bass (*Dicentrarchus labrax* L.) juveniles**

29

30 Luca Parma^{a*}, Manuel Yúfera^b, Carmen Navarro-Guillén^{b,1}, Francisco J. Moyano^c,
31 Matteo Soverini^d, Federica D'Amico^d, Marco Candela^d, Ramon Fontanillas^e, Pier Paolo
32 Gatta^a, Alessio Bonaldo^a

33

34 ^aDepartment of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra
35 50, 40064 Ozzano Emilia, Italy

36 ^bInstituto de Ciencias Marinas de Andalucía (ICMAN-CSIC), Apartado Oficial, 11519
37 Puerto Real, Cádiz, Spain

38 ^cDepartment of Biology and Geology, Universidad de Almería, La Cañada de San
39 Urbano, 04120 Almería, Spain

40 ^dUnit of Microbial Ecology of Health, Department of Pharmacy and Biotechnology,
41 University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

42 ^eSkretting Aquaculture Research Centre, Sjøhagen 3, 4016 Stavanger, Norway

43 ¹*Present address*: Centro de Ciências do Mar do Algarve (CCMAR), Universidade do
44 Algarve, Faro, Portugal.

45

46 **Corresponding author*: Luca Parma, Department of Veterinary Medical Sciences,
47 University of Bologna, Viale Vespucci 2, 47042 Cesenatico, FC, Italy. *Tel.*: +39 0547
48 338931; *Fax*: +39 0547 338941

49 E-mail address: luca.parma@unibo.it (L. Parma)

50 **Abstract**

51 Fishmeal (FM) possesses one of the highest buffering capacities in comparison to most
52 alternative vegetable aquafeed ingredients and its decreasing content in current
53 formulations might affect the ideal gastrointestinal environment for digestive enzyme
54 action and gut bacterial community of carnivorous fish species. A study was undertaken,
55 therefore, to assess growth response, gastrointestinal pH, digestive enzyme activity and
56 gut bacterial community of European sea bass (*Dicentrarchus labrax* L.) juveniles fed
57 low FM diets (10% FM) with or without a feed buffering additive, calcium carbonate
58 (FM10+B and FM10, respectively) in comparison to a standard FM diet (20% FM,
59 FM20). Three isonitrogenous and isolipidic extruded diets were fed to triplicate fish
60 groups of 80 individuals (initial weight: 23 g) to overfeeding over 64 days. No significant
61 differences due to low FM dietary levels were observed in final body weight, specific
62 growth rate, feed intake, feed and protein efficiency. Low FM diet did not affect
63 gastrointestinal pH in the stomach, anterior intestine, mid-intestine and hindgut at 0, 4, 8
64 and 12 hours post meal (hpm) while the inclusion of calcium carbonate in low FM diet
65 seems to slightly increase the pH in the hindgut at 12 hpm. The absence of significant
66 differences of pepsin, chymotrypsin, amylase and lipase activity suggests a comparable
67 digestive efficiency among treatments although trypsin activity was slightly reduced in
68 low FM diets after 4 hpm. Decreasing FM content seems to exert an effect on the overall
69 gut bacterial community analysed by next-generation sequencing even if no significant
70 effects on specific bacterial component were detected. The gut bacterial community in all
71 the treatments was particularly rich in lactic acid bacteria such as *Lactobacillus spp.*
72 which may provide important beneficial functions for the host and be associated with a
73 healthy intestinal epithelium. According to the results, increasing the feed buffering

74 capacity does not seem to improve digestive conditions while it is feasible to include 10%
75 FM dietary level in practical formulation for European sea bass juveniles without
76 negatively affecting growth, feed efficiency and digestive luminal conditions.

77

78 **Keywords**

79

80 European sea bass, fishmeal replacement, feed buffering capacity, gastrointestinal pH,
81 digestive enzyme, gut bacterial community

82

83 **1. Introduction**

84

85 In the issue of fishmeal (FM) replacement, a key area of investigation for continuing
86 to improve modern aquafeeds includes the evaluation of the effects of plant ingredients
87 and low FM diets on gastrointestinal luminal digestive conditions and gut microbiome.
88 Physiological values of pH in stomach and intestine allow adequate digestion of dietary
89 proteins and lipids by providing the optimal environment for the activation and action of
90 digestive enzyme. Optimal intestinal pH is also the ideal environment for gut microbial
91 communities and it is likely that an abnormal pH in the gut will alter microbial structure
92 and metabolism (Ilhan et al., 2017). Feed buffering capacity (BC) is the ability of a given
93 amount of feed to resist a change in pH after the addition of either an acidic or a basic
94 solution (Giger-Reverdin et al., 2002). Feed BC is a factor well known in livestock
95 production (Giger-Reverdin et al., 2002; Lawlor et al., 2005), since it can affect
96 gastrointestinal pH with consequences on protein digestion and gut microbiota in
97 monogastric vertebrates. In fish nutrition this is not a common issue since the dietary

98 inclusion of ingredients such as FM has usually guaranteed a high acid-binding capacity
99 of diets. FM has one of the highest buffering capacities in comparison to most alternative
100 vegetable aquafeed ingredients such as sunflower meal, soybean meal, rapeseed meal and
101 glutens (Giger-Revendin et al., 2002) and its decreasing content to relatively low levels
102 in current formulations might affect the ideal gastrointestinal pH for digestive enzyme
103 action and gut bacterial community of specific fish species. Different levels and models
104 of regulation of gastrointestinal pH in fish species of commercial interest have been
105 described. Species such as rainbow trout (*Oncorhynchus mykiss*) and cobia
106 (*Rachycentron canadum*), are characterised by a continuous acid secretion and the
107 maintenance of a low gastric pH during fasting while others displayed neutral gastric pH
108 during fasting followed by hydrochloric acid released only after the ingestion of a meal
109 (Bucking and Wood, 2009; Yúfera et al., 2019). Ranges in pH of several fish species have
110 been reported between 2.0 and 7.0 for the stomach and between 6.2 and 8.2 for the
111 intestine mainly depending on fish specific feeding habits including omnivorous vs.
112 carnivorous and frequent feeders vs. less frequent feeders (Krogdahl et al., 2015; Yúfera
113 et al., 2012). In this contest to the best of our knowledge little information is available for
114 the European sea bass (*Dicentrarchus labrax*) which seems to display very low pH value
115 in the stomach followed by alkaline levels in the intestine (Nikolopoulou et al., 2011). In
116 this carnivorous species a reduction of feed BC induced by a very low FM dietary level
117 might led to relatively low pH in the intestinal tract with consequences on feed utilisation.

118 Several studies have shown that European sea bass can cope well with high levels of
119 FM protein replacement by a mixture of plant feedstuff alone or in combination with other
120 terrestrial animal protein sources using different level of fish oil (FO). Kaushik et al.
121 (2004), during a 12 week experiment, reported no adverse consequence in terms of growth

122 or nitrogen utilization when using 5% FM and 20% FO employing soybean meal (SBM),
123 rapeseed meal, corn gluten meal and wheat gluten to replace FM in adult specimens (190-
124 330g). More recently, no negative effect on growth and feed intake in 67-200 g fish was
125 observed when 10% FM and 10% FO were used in a plant based diet for 118 days, even
126 though feed and protein utilisation was reduced in comparison to 20% and 30% FM diets
127 (Bonvini et al., 2018a). In juveniles (10-52 g), Torrecillas et al. (2017a, b) reduced both
128 FM and FO levels, down to 10 and 3% respectively, including plant and terrestrial animal
129 protein without affecting growth and feed utilization while a further FM reduction down
130 to 5% including 7% blood meal and 6% FO reduced feed intake and growth.

131 Hence, the aims of the present research were 1) to evaluate growth performance of
132 European sea bass juveniles fed a low FM diet (10% FM, 7% FO) as the sole animal
133 protein in comparison to a control diet (20% FM, 7% FO); 2) to evaluate the effects of
134 low FM diets on the gastrointestinal patterns of pH, digestive enzyme activities and gut
135 bacterial community; 3) to evaluate the effect of calcium carbonate dietary inclusion on
136 the gastrointestinal luminal digestive conditions and gut bacterial community.

137

138 **2. Materials and methods**

139

140 *2.1 Experimental diets*

141

142 Three isonitrogenous (47% protein) and isolipidic (16% lipid) diets were formulated,
143 two experimental diets with 10% FM with or without the inclusion of calcium carbonate
144 (CaCO₃) (FM10+B, FM10, respectively) and one control diet containing 20% FM
145 (FM20). CaCO₃ was added at 3% in FM10+B in order to achieve a higher buffering

146 capacity than FM10. Diets were formulated with FM and with a mixture of vegetable
147 ingredients currently used for European sea bass in aquafeed (Bonvini et al., 2018b). The
148 diets were produced via extrusion process by Skretting Aquaculture Research Centre,
149 Stavanger, Norway. The diameter of the pellet was 2 mm. Ingredients and proximate
150 composition of the experimental diets are presented in Table 1.

151

152 *2.2 Fish and rearing trial*

153

154 The experiment was carried out at the Laboratory of Aquaculture, Department of
155 Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European
156 sea bass juveniles were obtained from Panittica Italia (Torre Canne di Fasano, Brindisi,
157 Italy). At the beginning of the trial, 80 fish (initial average weight: 22.8 ± 0.7 g) per tank
158 were randomly distributed into nine 900 L square tanks with a conical base. Each diet
159 was administered to triplicate groups, assigned in a completely random manner, over 64
160 days. Tanks were provided with natural seawater and connected to a closed recirculation
161 system (overall water volume: 13 m^3). The rearing system consisted of a mechanical sand
162 filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25 mJ/cm^2 : $32 \text{ m}^3 \text{ h}^{-1}$,
163 Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain).
164 The water exchange rate within each tank was 100% every hour, while the overall water
165 renewal amount in the system was 5% daily. During the trial, the temperature was kept at
166 22 ± 1.0 °C and the photoperiod was maintained at 12 h light and 12 h dark through
167 artificial light. The oxygen level was kept constant ($8.0 \pm 1.0 \text{ mg L}^{-1}$) by a liquid oxygen
168 system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy).
169 Ammonia (total ammonia nitrogen $\leq 0.1 \text{ mg L}^{-1}$), nitrite ($\leq 0.2 \text{ mg L}^{-1}$) and salinity (25 g

170 L⁻¹) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab
171 business, Darmstadt, Germany). Sodium bicarbonate was added on a daily basis to keep
172 pH constant at 7.8–8.0. Feed was provided to satiation by oversupplying the feed via
173 automatic feeders by approximately 10% of the daily ingested ration, twice a day (8:30,
174 16:30) for six days a week, while one meal was supplied on Sundays, as reported by
175 Mongile et al. (2014). Each meal lasted 1 hour, after which the uneaten pellets of each
176 tank were gathered, dried overnight at 105°C, and their weight was deducted for overall
177 calculation.

178

179 *2.3 Sampling*

180

181 At the beginning and at the end of the experiment, all the fish in each tank were
182 anaesthetised by 2-phenoxyethanol at 300 mg L⁻¹ and individually weighed. Specific
183 growth rate (SGR), feed intake (FI) and feed efficiency (FE) were calculated. The
184 proximate composition of the carcasses was determined at the beginning of the trial on a
185 pooled sample of 10 fish and on a pooled sample of 5 fish per tank at the end of the trial.
186 Protein efficiency rate (PER) and gross protein efficiency (GPE) were calculated.
187 Furthermore, at the end of the trial, wet weight, viscera and liver were individually
188 recorded for 5 fish per tank to determine viscerosomatic index (VSI) and hepatosomatic
189 index (HSI). At the end of the trial, three fish per tank were sampled at 0, 4, 8 and 12
190 hours post meal (hpm) to measure gastrointestinal pH. The meal was supplied to satiation
191 in keeping with the standard procedures employed during the trial. In addition at 4 and 8
192 hours post-prandial samples of the whole gastrointestinal tract (n=4/treatment) were
193 stored at –80 °C and then freeze-dried for enzyme activity analysis. At the end of the trial

194 at 12 hpm samples of hindgut content from 3 fish per tank were collected individually for
195 gut bacterial community characterization. The gastrointestinal tract was dissected under
196 sterile conditions and the hindgut content was squeezed out into an Eppendorf tube (one
197 per fish) and placed at -80°C until DNA extraction (Parma et al., 2016). Hindgut was
198 defined as the section from the ileorectal valve to the anus. Gut bacterial community
199 analyses were then performed on a pooled of 300 mg of intestinal content originating
200 from the 3 fish sampled for each tank (100mg of intestinal content from each individual
201 fish sampled) according to Parma et al. (2016).

202 All experimental procedures were evaluated and approved by the Ethical-Scientific
203 Committee for Animal Experimentation of the University of Bologna, in accordance with
204 European directive 2010/63/UE on the protection of animals used for scientific purposes.

205

206 *2.4 Calculations*

207

208 The formulae employed were as follows:

209 Specific growth rate (SGR) ($\% \text{ day}^{-1}$) = $100 * (\ln \text{FBW} - \ln \text{IBW}) / \text{days}$ (where FBW and
210 IBW represent the final and the initial body weights). Feed Intake (FI) (g kg ABW^{-1}
211 day^{-1}) = $((1000 * \text{total ingestion}) / (\text{ABW})) / \text{days}$) (where average body weight, $\text{ABW} =$
212 $(\text{IBW} + \text{FBW}) / 2$. Feed efficiency (FE) = wet weight gain/dry feed intake. Viscerosomatic
213 index (VSI) (%) = $100 * (\text{viscera weight} / \text{body weight})$. Hepatosomatic index (HSI) (%)
214 = $100 * (\text{liver weight} / \text{body weight})$. Protein efficiency rate (PER) = $(\text{FBW} - \text{IBW}) /$
215 protein intake. Gross protein efficiency (GPE) (%) = $100 * [(\% \text{ final body protein} * \text{FBW})$
216 $- (\% \text{ initial body protein} * \text{IBW})] / \text{total protein intake fish}$.

217

218 *2.5 Gastrointestinal luminal pH measurements*

219

220 After an overdose of anaesthetic with 2-phenoxyethanol (1g L^{-1}), fish were dissected
221 to make the digestive tract accessible. Measurements were taken using a pH meter (Orion,
222 Star A221) equipped with a microelectrode following the procedure described in Yúfera
223 et al. (2012). In short, the tip of the microelectrode (diameter 1.7 mm) was inserted in the
224 gastrointestinal lumen through small slits made in the stomach, anterior intestine, mid-
225 intestine and hindgut. Hindgut was defined as the section from the ileorectal valve to the
226 anus. Instrument calibration using standard buffering solutions at pH 4, 7 (Crison) was
227 performed before each fish measurement and the microelectrode was carefully cleaned
228 before each measurement.

229

230 *2.6 Enzyme activity analysis*

231

232 Enzyme extracts for enzyme activity measurement were prepared from samples
233 previously freeze-dried. Stomach and intestine were dissected and homogenised
234 separately. Samples were manually homogenised in 5 mL distilled water and centrifuged
235 for 10 min at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany) at 4°C. The
236 supernatants from stomach samples were used for pepsin activity measurement, and the
237 supernatants from intestine samples were used for the analysis of trypsin, chymotrypsin,
238 amylase and lipase activities.

239 Pepsin activity was determined by the method of Anson (1938): 15 μL of extracts were
240 mixed to 1 mL of 0.5% acid-denatured bovine haemoglobin diluted in 0.2 M HCl-Glycine
241 buffer. With the objective of measuring active pepsin, for each sample the buffer pH was

242 adjusted to the measured pH of the sample. After incubation at 25°C for 30 min, the
243 reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled at 4°C
244 for 15 min and then centrifuged at 12000 rpm for 15 min. The absorbance of the resulting
245 supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme
246 extracts just after TCA to the reaction mixture. A standard curve was prepared with
247 varying concentrations of tyrosine to determine the molar extinction coefficient of
248 tyrosine at 280 nm. One unit of activity was defined as the amount of enzyme required to
249 produce 1 µg of tyrosine per minute.

250 For alkaline protease activities, trypsin activity was assayed using BAPNA (N-
251 benzoyl-DL-arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as substrate. 0.5 mM
252 BAPNA was dissolved in 1mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL
253 with Tris-HCl 50mM, pH 6.5, containing 20 mM CaCl₂. Chymotrypsin activity was
254 determined using 0.2 mM SAPNA (N-succinyl-L-Ala-L-Pro-Phe-p-nitroanilide)
255 dissolved in 1mL DMSO and then made up to 100 mL in the same buffer. Reactions were
256 started in 96-well microplate by the addition of 15 µL of the enzyme extract to 200 µL of
257 respective substrate and liberation of p-nitroaniline was kinetically followed at 405 nm
258 in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA).

259 Lipase activity was measured following the method described by Versaw et al. (1989),
260 with some modifications. The assay mixture contained: 60 µL of 100 mM sodium
261 taurocholate, 540 µL of 50 mM Tris-HCl, pH 6.5, 10 µL of enzyme extract and 6 µL of
262 β-Naphthyl caprilate. The reaction was maintained for 25 min at 25°C and after this time
263 6 µL of 100 mM Fast Blue BB in DMSO were added and incubated at 25°C for 5 min.
264 The reaction was then stopped with 60 µL TCA 0.72 N. Finally, 815 µL of 1:1 (v:v) ethyl
265 acetate/ethanol solution was added and absorbance recorded at 540 nm. A standard curve

266 was prepared by replacing β -naphthyl caprylate by varying concentrations of β -naphthol
267 (N1250, Sigma-Aldrich) dissolved in DMSO. One unit of activity was defined as the
268 amount of enzyme required to produce 1 μ mol of β -naphthol per minute.

269 Amylase activity was determined by the 3, 5-dinitrosalicylic acid (DNS) procedure
270 (Bernfeld, 1955), using 2% soluble starch as substrate. 30 μ L of enzyme extract and 300
271 μ L of substrate were incubated at 37°C for 30 min. The reaction was stopped by addition
272 of 150 μ L DNS and was heated in boiling water for 10 min. Then, after cooling in ice,
273 1.5 mL of distilled water was added to the mixture and absorbance was measured at 540
274 nm. Blanks were constructed by adding the enzyme extracts just after DNS to the reaction
275 mixture. A standard curve was prepared by replacing starch by varying concentrations of
276 maltose dissolved in water. One unit of activity was defined as the amount of enzyme
277 required to produce 1 μ g of maltose per minute.

278 In all cases, enzyme activities were provided as activity units per wet weight unit of
279 fish to prevent variability associated with fish size and gut content.

280

281 *2.7 Gut bacterial community DNA extraction and sequencing*

282

283 Total bacterial DNA was extracted from a pool of hindgut content obtained from 3 fish
284 per tank (100 mg of hindgut content per fish) for a total of 9 tanks, as also previously
285 reported in Parma et al. (2016). Afterwards, the V3–V4 hypervariable region of the 16S
286 rRNA gene was amplified using the 341F and 785R primers (Klindworth et al., 2013)
287 with added Illumina adapter overhang sequences and 2x KAPA HiFi HotStart ReadyMix
288 (KAPA Biosystems) (Turrone et al., 2016). Briefly, the thermal cycle consisted of an
289 initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing

290 at 55°C for 30 s and extension at 72°C for 30 s, and a final extension step at 72°C for 5
291 min (Parma et al., 2016). PCR reaction were cleaned up for sequencing by using
292 Agencourt AMPure XP magnetic beads as recommended in the Illumina protocol “16S
293 Metagenomic Sequencing Library Preparation” for the MiSeq system, and as used in
294 several other publications (Biagi et al., 2018; Soverini et al., 2016). Raw sequences were
295 processed using the QIIME pipeline (Caporaso et al., 2010). After length
296 (minimum/maximum = 200/600 bp) and quality filtering with default parameters, reads
297 were binned into OTUs at a 0.97 similarity threshold using UCLUST (Edgar, 2010).
298 Assignment was carried out by using the RDP classifier against Greengenes database
299 (May 2013 version). Alpha-diversity analysis was performed using the observed OTUs,
300 Faith's PD, Chao1, Shannon index metrics and the species evenness calculated as the ratio
301 between the Shannon index and the natural logarithm of species richness (Observed
302 OTUs index). Beta-diversity was estimated by Bray-Curtis distances, which were used as
303 input for principal coordinates analysis (PCoA).

304

305 *2.8 Analytical methods*

306

307 Diets and whole body were analysed for proximate composition. Moisture content was
308 obtained by weight loss after drying samples in a stove at 105 °C until a constant weight
309 was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl
310 method and multiplying N by 6.25. Total lipids were determined according to Bligh and
311 Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant
312 weight in a muffle oven at 450 °C. Gross energy was determined by a calorimetric bomb
313 (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, U.S.A).

314 Feed BC was determined as the amount of HCl needed to lower the pH solution to pH
315 3 in a titration; procedures followed the methods proposed by Márquez et al. (2012). The
316 procedure consisted in suspending 1 g of the diet in 5 mL of distilled water, keeping it
317 magnetically stirred, then adding successive aliquots of HCl 1 N and recording the pH 30
318 s after each addition of HCl. All the measurements were made using the same pH meter
319 adopted to measure the gastrointestinal pH (Orion, Star A221, equipped with a
320 microelectrode) presented in M&M at 2.5. Feeds BC is included in table 1.

321

322 *2.9 Statistical analysis*

323

324 All data are presented as mean \pm standard deviation (SD). A tank was used as the
325 experimental unit for analysing growth performance, and a pool of five sampled fish was
326 considered the experimental unit for analysing carcass composition, whereas individual
327 fish were used for analysing VSI, HSI, pH and enzyme activity. Data of growth
328 performance, VSI, HSI, nutritional indices and enzyme activity were analysed by a one-
329 way analysis of variance (ANOVA) and in case of significance ($P \leq 0.05$) Tukey's post
330 hoc test was performed. The normality and/or homogeneity of variance assumptions were
331 validated for all data preceding ANOVA. Student's T-test was adopted to determine
332 difference of enzyme activity within the same treatment at different sample time. Data of
333 gastrointestinal pH as function of time and diet were analysed by Two-way ANOVA
334 followed by Tukey's post hoc test. The R packages "Stats" and "Vegan" were used to
335 perform gut microbiota statistical analysis. In particular, to compare the microbiota
336 structure among different groups for alpha and beta-diversity, a Mann-Whitney-Wilcoxon
337 test was used while data separation in the PCoA was tested using a permutation test with

338 pseudo-F ratios (function “Adonis” in the “Vegan” package). Statistical analyses were
339 performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA,
340 USA) and RStudio interface for R (<https://www.r-project.org>). The differences among
341 treatments were considered significant at $P \leq 0.05$.

342

343 **3. Results**

344

345 *3.1 Growth*

346

347 Growth performances are summarised in Table 2. No significant differences were
348 observed after 64 days in terms of growth performance (final body weight and SGR), feed
349 intake (FI) and feed efficiency (FE). Data on biometric indices, body composition and
350 nutritional indices are shown in Table 3. HSI values were significantly lower in FM10
351 compared to FM20, while no significant differences were found in VSI. Furthermore, no
352 significant differences were found for whole body composition and for the nutritional
353 indexes (PER and GPE).

354

355 *3.2 Gastrointestinal luminal pH pattern*

356

357 Postprandial changes in the gastrointestinal luminal pH are shown in Fig. 1. No significant
358 differences between diets at each sampling time were detected in the pH of the stomach.
359 pH in the stomach decreased at 4 hpm with a similar pattern for all the treatments reaching
360 lower levels at 8 hpm and resulting in the range of 2.53-2.83. Specifically, in FM10 and
361 FM20 pH significantly declined between 0 and 4 hpm while no significant differences

362 between the same times were detected in FM10+B. Afterwards gastric pH remained
363 generally stable (below 4) (Fig. 1A). No significant differences between diets at each
364 sampling time were detected in the pH of the anterior intestine. pH displayed stable values
365 ranging from 5.72 to 6.04 until 4 hpm; thereafter at 8 hpm in FM20 the values decreased
366 to 5.0 while at 12 hpm, pH resulted in the range of 5.53-6.07 for all the treatments (Fig.
367 1B).

368 No significant differences between diets at each sampling time were detected in the
369 pH of the mid-intestine which showed a general fluctuating pattern (5.48-6.74) with
370 values decreasing below 6 at 8 hpm for all the treatments followed by an increasing
371 pattern at 12 hpm in FM20 and FM10+B (Fig. 1C). pH values in the hindgut ranged from
372 5.29 to 6.59. Data recorded in FM10 were significantly lower at 12 hpm compared to
373 FM10+B. In FM10 pH displayed stable values between 8 and 12 hpm while at the same
374 time an increasing trend was observed in FM20 and FM10+B (Fig. 1D).

375

376 *3.3 Digestive enzymes activity*

377

378 The results of pepsin, trypsin, chymotrypsin, amylase and lipase activities measured
379 in the different experimental groups are shown in Table 4. No significant differences
380 among treatments at each sampling time were detected in the pepsin, chymotrypsin,
381 amylase and lipase activity while at 4 hpm trypsin displayed a significant difference
382 ($P=0.0452$), being significantly higher in FM20 compared to the other treatments.

383

384 *3.4 Gut bacterial community profiles*

385

386 In Figure 2 the composition of the gut bacterial ecosystem is shown at phylum (A) and
387 family (B) level subdivided by individual tank and treatment. The gut microbial
388 ecosystem of the European sea bass was dominated at the phylum level by *Firmicutes*
389 (average abundance 74.2%), followed by subdominant phyla such as *Proteobacteria*
390 (12.3%), *Cyanobacteria* (7.2%) and *Actinobacteria* (5.1%). The structure of the microbial
391 ecosystem was more complex when was observed at the family level, where a dominance
392 of the *Lactobacillaceae* family (38.0%) was reported, followed by *Leuconostocaceae* (9.8
393 %) and *Streptococcaceae* (9.1%).

394 Different metrics have been utilized to calculate alpha-diversity, including observed
395 OTUs, Faith's PD, Chao1, Shannon index and species evenness (Table 5). No significant
396 differences in the gut bacterial community alpha-diversity according to the different diets
397 were detected, however different diets showed a tendency to produce a different evenness
398 within the bacterial species with a lower value in FM20 and higher values for both FM10
399 diets. (Table 5). In Supplementary Table 1 the average gut microbial abundance, resulting
400 from the three different diets is shown. Although no specific bacterial component has
401 undergone a statistically significant change in the relative abundance between dietary
402 regimens (p value > 0.05, wilcoxon test), it is possible to observe a tendency in the
403 decrease of *Lactobacillaceae* abundance in both FM10 diets if compared to the FM20
404 control diet. PCoA plot based on the Bray-Curtis distances revealed a significant
405 separation of the 3 different nutritional groups (Figure 3). Further, Adonis
406 PERMANOVA ('vegan' package of RStudio) revealed a significant separation between
407 groups in the multidimensional space (p value 0.02). In detail, diet FM20 was
408 significantly separated both from diet FM10+B (p value 0.017) and FM10 (p value 0.005).
409 Groups FM10+B and FM10 showed a non-significant separation (p value 0.09). Figure 4

410 showed the relative abundance of the most represented bacteria genera belonging to
411 Lactic acid bacteria (LAB). In particular, *Lactobacillus* showed a decrease and
412 comparable values in both FM10 diets.

413

414 **4. Discussion**

415

416 Several studies have recently investigated the utilization of low FM diets in European
417 sea bass and their effects on growth, gut morphology, fillet quality and plasma
418 biochemistry, while data concerning the effects on luminal digestive conditions and gut
419 microbiota are scarce. In the present study, fish fed 10% FM level as the sole animal
420 protein source showed equal growth, feed and protein utilization in comparison to a
421 control 20% FM diet. The growth performances registered in this trial are similar to or
422 even higher than those recently reported on European sea bass juveniles (7-28 g) fed
423 vegetable protein-based diets containing 8% and 2% (on dry weight basis) of FM and
424 soluble fish protein concentrate, respectively with a FO level of 15% (Coutinho et al.,
425 2017). Similar findings were also reported by Torrecillas et al. (2017a) which reduced
426 both FM and FO levels to 10 and 3% respectively in practical, commercially
427 manufactured feeds, without affecting growth and feed utilization of European sea bass
428 juveniles. However, in this latter study 6% of blood meal was also employed to replace
429 FM, while in the present study FM was the sole animal protein included. A further FM
430 reduction down to 5%, including also 7% blood meal (spray dried), soy protein
431 concentrate, rapeseed meal, glens and a 6% of FO was tested in European sea bass
432 juveniles of similar size (20-68g) and using similar rearing conditions of the present study
433 (Torrecillas et al., 2018). The authors needed 90 days to cover that growth period

434 reporting a SGR of 1.40 while in the present study the trial lasted 64 days with a SGR of
435 1.76 (FM10). The data obtained in European sea bass juveniles in the present study go
436 beyond the results of a recent study by Bonvini et al. (2018b) where a trend towards lower
437 growth performance at higher FM replacement levels was observed when 10% FM diet
438 was compared to 20 and 30% FM diets using exclusively plant-derived ingredients to
439 replace FM and using 10% FO level. However Bonvini et al. (2018b) employed bigger
440 fish (70-200g) than the present study and the most marked differences in terms of FCR
441 and protein efficiency were detected when comparing 10% with 30% FM diets.

442 Low FM dietary level might reduce feed BC with possible effects on gastrointestinal
443 luminal digestive conditions. In the present study, gastric pH was not significantly
444 affected by 10% FM dietary level in comparison to a control diet. Our results confirm
445 previous observations that European sea bass maintain a gastric pH slightly acidic (4.5-
446 5) during fasting followed by a strong acidification (pH below 3) stimulated by the
447 ingestion of food (Nikolopoulou et al., 2011). However, the authors reported the lowest
448 gastric pH values (3-3.5) at 12 hpm while in the present study the levels dropped to 3
449 already at 4 hpm to reach the lowest values (2.5) at 8 hpm. Since the authors utilised a
450 higher FM dietary level compared to the present study (40 to 60% FM dietary level) an
451 expected higher feed BC might have determined those differences. The cause of the
452 variation in gastrointestinal pH in the same species between experiments could also be
453 related to fish size/age due to changes in feeding habit from juvenile to adults, which can
454 affect digestion controls (Yúfera et al., 2004). However, no significant differences in
455 gastrointestinal pH values have been reported from 7 up to 100 g sea bream juveniles and
456 from 0.3 to 10 kg Atlantic salmon (*Salmo salar*) (Yúfera et al., 2004; Krogdahl et al.,
457 2015). Focusing on the effects of the CaCO₃ inclusion at gastric level, the pH pattern of

458 FM10+B did not show a significant decreasing trend between 0 and 4 hpm (displayed in
459 the other treatments) indicating a possible feed BC effect able to slightly resist gastric
460 acidification during the first hours after a meal. Data on intestinal pH displayed neutral
461 to slightly acidic values indicating an effective active pancreatic exocrine HCO₃ secretion
462 in the anterior intestinal tract as previously reported by Nikolopoulou et al. (2011) for this
463 species fed one morning meal. However, these authors found an increasing tendency over
464 time with most intestinal pH values ranging between 7 and 8. Thus, the higher capability
465 of rapid alkalinisation at the intestinal level mentioned by them was less evident in the
466 present study. Probably, the two meals offered in the present study altered the feed transit
467 time and therefore the luminal pH, as it was observed in gilthead sea bream at different
468 moments or different daily feeding frequencies (Montoya et al., 2010; Yúfera et al.,
469 2014). While experimental diets did not affect pH values in the anterior and mid-intestine,
470 the addition of CaCO₃ in low FM diet led to a higher pH in the hindgut at 12 hpm
471 compared to FM10. This difference is also reinforced by the different temporal pH pattern
472 occurring in FM10 (no significant differences between 8 and 12 hpm) and FM10+B
473 (significant pH increasing between 8 and 12 hpm) for both mid-intestine and hindgut
474 indicating that the higher feed BC of FM10+B seems to facilitate the increase of pH
475 between 8 and 12 hpm in the mid-intestine and hindgut.

476 Overall results for digestive enzymes measured at 4 and 8 hpm indicated a similar
477 enzyme activation under the different treatments at each sample time, while higher values
478 of trypsin, amylase and lipase were reported at 4 hpm compared to 8 hpm. Since 4 hpm
479 corresponds to the beginning of the transit of feed from the stomach to the intestine and
480 no feed is expected in the hindgut at this time (Bonvini et al., 2018b), the results might
481 indicate a predominant function of these enzymes in the anterior intestine as already

482 described in European sea bass and other fish species (Tibaldi et al., 2006, Magalhães et
483 al., 2015, Yaghoubi et al., 2016). No evidence of any relation among the pH patterns and
484 enzyme activities could be observed, probably due to the low amplitude of the differences
485 in pH among treatments which also occurred mainly in the hindgut where a low enzyme
486 activity is also expected. Despite similar overall results in digestive enzyme activity
487 among treatment, trypsin activity at 4 hpm was slightly reduced in low FM diets compared
488 to the control diet. The presence of residual protease inhibitors in vegetable aquafeed
489 ingredients affecting enzyme activity has been previously described in other fish species
490 (Krogdahl et al., 2003, Santigosa et al., 2008, Yaghoubi et al., 2016) and according to
491 Moyano et al. (1999) it depends on the type and amount of plant meal, extension of the
492 feeding period and sensitivity of fish species. Few data are available for European sea
493 bass fed a very low FM diet. However Tibaldi et al. (2006) reported no clear evidence of
494 the selective soybean meal (SBM) induced disturbances of the digestive–absorptive
495 functions in the intestine of European sea bass when fed dietary SBM levels up to 53%.
496 Similarly, Magalhães et al. (2015) did not find significant alteration in intestinal protease
497 activity in the same species fed by corn distillers dried grains with solubles at 30% dietary
498 level. In the present study these differences could be attributed to a delay in the enzyme
499 activation; however their low amplitude ($P= 0.045$) together with the comparable feed
500 and protein efficiency achieved, suggest the absence of a digestive disturbance.

501 According to our findings, the gut bacterial community is widely dominated by
502 *Firmicutes*, followed by *Proteobacteria*, *Cyanobacteria* and *Actinobacteria*. At family
503 level the gut bacterial community was mainly represented by *Lactobacillaceae*,
504 *Leuconostocaceae* and *Streptococcaceae*. Our data differ from previous pyrosequencing
505 surveys of the gut bacterial community in European sea bass fed functional diets (Carda-

506 Diéguez et al., 2014). The authors by analysing the microbial composition of intestinal
507 mucosa showed a dominance of the family *Bradyrhizobiaceae* (*Proteobacteria*),
508 *Porphyromonadaceae* (*Bacteroidetes*) and *Burkholderiaceae* (*Proteobacteria*) in fish fed
509 a control diet or functional diets including β -glucans and essential oils, respectively. Also
510 Gatesoupe et al. (2016), based on pyrosequencing analysis from 16S rRNA, found
511 *Proteobacteria* as the dominant phyla (94.4%) followed by *Bacteroidetes*, *Actinobacteria*
512 and *Firmicutes* as subdominant components of the gut microbiota of European sea bass.
513 Both works characterised the bacteria communities that adhere to the mucosal surface,
514 the autochthonous microbiota, (Ringo et al., 2018), which could differ from the
515 microbiota of the gastrointestinal lumen (the allochthonous microbiota) which was the
516 focus of the present study. In addition, to the best of our knowledge, data reporting the
517 characterization of the gut bacterial community from the intestinal lumen by next-
518 generation sequencing (NGS) in European sea bass juveniles are scarce. No significant
519 differences on bacterial alpha-diversity was observed in response to the dietary changes
520 which is in agreement with other carnivorous fish species where a FM replacement with
521 a mix of plant proteins or terrestrial animal protein did not induce significant changes in
522 gut microbial alpha-diversity indices (Apper et al., 2016; Parma et al., 2016; Rimoldi et
523 al., 2018). The lack of effect on diversity could be considered beneficial, since a reduction
524 in diversity may provide less competition for opportunistic or invading pathogens which
525 may enter the gastrointestinal tract of fish via feed or water (Apper et al., 2016). However,
526 although not significant, an increasing evenness was observed in both 10% FM diets
527 compared to 20% FM diet indicating a more homogeneous distribution of the abundances
528 of the different microbial players involved. These results may suggest an increase of
529 microbial diversity at low FM diet, in agreement with previous findings indicating that

530 feed and feeding habit is an important factor influencing gut microbial diversity of fish
531 species with an increasing trend of diversity following the order of carnivores, omnivores
532 and herbivores (Wang et al., 2018).

533 Our findings showed significant differences in the overall gut bacterial composition
534 between the control diet and the low FM diets as shown by PCoA plot based on the Bray-
535 Curtis distances. The effect of FM substitution on gut microbiota has been also
536 investigated in other carnivorous fish species. The dominance of *Firmicutes* we observed
537 in the European sea bass is in agreement to that found in sea bream fed high FM
538 replacement diet (FM 15%) using similar aquafeed plant ingredients employing in the
539 present study (soy-derivates, corn glutens, wheat gluten and wheat meal) (Parma et al.,
540 2016). In the same work a FM reduction from 35% to 15% did not exert an impact on the
541 overall gut bacterial community even if the inclusion up to 30% SBM seemed to
542 positively favor taxa of the *Lactobacillaceae* family. Similarly, in Asian sea bass (*Lates
543 calcarifer*) an increase of reads of some beneficial LAB (*Firmicutes*) in the alloctonous
544 gut microbiota, such as *Leuconostoc*, was observed when FM was replaced by a mix of
545 animal and plant proteins (Apper et al., 2016). Accordingly, in rainbow trout several
546 studies revealed that plant ingredients in the diet were often associated with a higher
547 *Firmicutes:Proteobacteria* ratio in comparison to FM-based diet, which favoured instead,
548 the presence of Proteobacteria. At this regard, Rimoldi et al. (2018) found that the dietary
549 inclusion of at least 25% of plant proteins favoured the presence of genera from the
550 *Firmicutes* phylum regardless of the content of animal proteins. Interestingly, the authors
551 stated that intestinal microbiome of rainbow trout which also showed the worst
552 performances in terms of growth and feeding efficiency, was characterized by scarce
553 amount of LAB and by low abundance of *Clostridiales*. In contrast, in sea bream Estruch

554 et al. (2015) found a reduction on the relative abundance of *Firmicutes* (*Streptococcus*
555 and *Lactococcus*) and an increase of Proteobacteria when FM was totally replaced by a
556 plant protein diet (58% FM diet vs 0 % FM diet). Similarly, in the carnivorous northern
557 snakehead (*Channa argus*) the inclusion of high level of dietary plant ingredient (70%
558 SBM, 15% FM) led to an increase of Proteobacteria (including opportunistic pathogens)
559 and exerted an adverse effect on the relative abundance of *Firmicutes*, including the
560 beneficial *Lactococcus*, *Geobacillus*, *Streptococcus* and *Bacillus*. However, in both
561 Estruch et al. (2015) and Miao et al. (2018) the decrease in *Firmicutes* and increase in
562 *Proteobacteria* was not only associated to the high level of FM replacement by dietary
563 plant protein but also to a lower survival rate and growth performance, respectively, in
564 comparison to their control FM dietary treatment.

565 In the present study, although no specific bacterial component has undergone a
566 significant difference between dietary regimens, *Firmicutes:Proteobacteria* ratio was
567 higher in FM10 (8.9) compared to both FM10+B (4.9) and FM20 (5.3) (data not shown).
568 If we compare the FM10 and FM20 the differences may be related to the reduction of
569 FM, while lower gastrointestinal pH could have been responsible of the differences
570 concerning FM10 and FM10+B which resulted in the same dietary FM and plant protein
571 composition but displayed different pH in the hindgut. Intestinal pH could play a role in
572 bacterial growth, and the values recorded in the present study (hindgut pH 5.29-6.59) may
573 have favoured the growth and dominance of LAB (*Firmicutes*) which have their optimum
574 generally at 5.5-5.8 (Ringo et al., 2018).

575 Genera belonging to LAB were the most represented in all the treatments accounting
576 for up to 50% of the total bacterial abundance. In particular *Lactobacillus spp.* displayed
577 higher abundance in FM20 compared to the other diets.

578 LAB are considered to be the most promising bacterial genera as probiotic in
579 aquaculture due to their ability to stimulate the host gastrointestinal development,
580 digestive function, mucosal tolerance and immune response, and to improve disease
581 resistance, even if the underlying mechanism is still poorly understood (Ringo et al.,
582 2018). On the contrary, it should be mentioned that in addition to the numerous beneficial
583 LAB, there are also several pathogenic species for fish within LAB's genera (Ringo et
584 al., 2018). What favours the presence of LAB in fish gut is still under debate. However,
585 some studies indicate the presence of feed plant derivate containing indigestible fibre and
586 oligosaccharides as the primary cause because LAB utilise such substrates for their
587 metabolism and growth in sea bream and Atlantic salmon (Parma et al., 2016, Gajardo et
588 al., 2017). Accordingly, the high level of plant ingredient of the present study may have
589 favoured a 50% abundance of LAB. Furthermore, as previously discussed, the high
590 abundance of LAB found in all treatments which all exhibited optimal growth and feed
591 utilization, may be also indicative of optimal gut health condition.

592

593 **Conclusion**

594

595 In conclusion, 10% FM dietary level as the sole animal protein of practical plant-based
596 diets led to equal growth and feed utilization in comparison to 20% FM dietary level. Low
597 FM diet did not affect gastrointestinal pH in the stomach, anterior intestine, mid-intestine
598 and hindgut, while the inclusion of calcium carbonate in low FM diet seems to slightly
599 increase the pH in the hindgut. Results of digestive enzyme activities indicated a
600 comparable digestive efficiency among treatments although trypsin activity was slightly
601 reduced in low FM diets after 4 hours post meal. Based on the present results decreasing

602 FM content seems to exert an effect on the overall gut bacterial community analysed by
603 NGS even if no significant effects on specific bacterial component were detected. The
604 gut bacterial community in all the treatments was particularly rich in lactic acid bacteria
605 such as *Lactobacillus spp.* which may provide important beneficial functions for the host
606 and be associated with a healthy intestinal epithelium. According to the results, increasing
607 the feed buffering capacity does not seem to promote digestive conditions while it is
608 feasible to include 10% FM dietary level in practical formulation for European sea bass
609 juveniles without negatively affecting growth, feed efficiency and digestive luminal
610 conditions.

611

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613

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616

617 **References**

618

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Table 1. Ingredients, proximate composition and buffering capacity of the experimental diets

	FM20	FM10	FM10+B
<i>Ingredients, % of the diet</i>			
Fish meal	20.0	10.0	10.0
Soybean meal	7.20	7.00	4.90
Soya concentrate	25.0	30.0	30.0
Wheat gluten	8.16	15.45	16.96
Corn gluten	7.00	2.01	3.58
Wheat	20.95	22.89	19.04
Fish oil	6.89	7.47	7.38
Rapeseed oil	4.59	4.98	4.92
Vit/Min premix ¹	0.20	0.20	0.20
CaCO ₃	0.0	0.0	3.0
Lysine	-	0.11	0.11
Methionine	-	0.36	0.36
<i>Proximate composition, % on a wet weight basis</i>			
Moisture	5.83	5.76	5.67
Protein	47.1	46.6	46.6
Lipid	16.9	15.6	16.1
Ash	5.77	5.15	7.30
Gross energy, cal g ⁻¹	4975.7	4985.4	4923.1
pH	6.4	6.4	6.5
Buffering capacity ²	0.75	0.53	0.75

¹ Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011)

² Amount (ML) 1 N HCL to lower 1 g of the diet to pH 3

CaCO₃ = calcium carbonate

FM = fishmeal

B = buffering additive

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Table 2. Growth performance and feed intake of European sea bass juveniles fed the experimental diets over 64 days.

	<i>Experimental diets</i>			<i>P</i> value
	FM20	FM10	FM10+B	
IBW (g)	22.7 ± 1.02	22.9 ± 0.06	22.9 ± 0.96	0.943
FBW (g)	67.9 ± 1.28	70.7 ± 2.39	66.0 ± 3.99	0.195
SGR (% day ⁻¹)	1.71 ± 0.05	1.76 ± 0.05	1.65 ± 0.03	0.073
FI (g kg ABW ⁻¹ day ⁻¹)	20.5 ± 0.4	20.0 ± 0.9	19.8 ± 0.4	0.380
FE	0.81 ± 0.02	0.85 ± 0.05	0.81 ± 0.03	0.495
Survival %	100 ± 0.0	100 ± 0.0	99.6 ± 0.72	0.422

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

FM20 = 200g kg⁻¹ fishmeal (FM); FM10 = 100g kg⁻¹ FM; FM10+B = 100g kg⁻¹ FM + 30g kg⁻¹ buffer (B, CaCO₃).

IBW = Initial body weight.

FBW = Final body weight.

SGR = Specific growth rate (% day⁻¹) = 100 * (ln FBW - ln IBW) / days.

ABW = average body weight = (IBW + FBW)/2.

FI = Feed intake (g kg ABW⁻¹ day⁻¹) = ((1000*total ingestion)/(ABW))/days).

FE = Feed efficiency = wet weight gain/dry feed intake

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Table 3. Biometric indices, body composition and nutritional indices of European sea bass juveniles fed the experimental diets

	Experimental diets			<i>P</i> value
	FM20	FM10	FM10+B	
Biometric indices				
VSI	12.3 ± 1.3	11.9 ± 1.4	12.2 ± 1.3	0.772
HSI	2.7 ^b ± 0.5	2.2 ^a ± 0.4	2.4 ^{ab} ± 0.5	0.044
Whole body composition, %				
Protein	17.3 ± 0.6	16.7 ± 0.2	17.2 ± 0.4	0.432
Lipid	17.0 ± 0.8	17.6 ± 0.1	16.8 ± 1.4	0.571
Ash	3.51 ± 0.24	3.43 ± 0.02	3.56 ± 0.20	0.670
Moisture	62.1 ± 0.8	61.9 ± 0.3	61.8 ± 0.5	0.834
Nutritional indices				
PER	1.62 ± 0.04	1.71 ± 0.11	1.65 ± 0.06	0.385
GPE	27.3 ± 1.92	27.9 ± 1.38	28.0 ± 1.94	0.856

Data are given as the mean (n=3; n=15 for VSI, HSI) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

FM20 = 200g kg⁻¹ fishmeal (FM); FM10 = 100g kg⁻¹ FM; FM10+B = 100g kg⁻¹ FM + 30g kg⁻¹ buffer (B, CaCO₃)

VSI = Viscerosomatic index (%) = 100*(viscera weight/final body weight, FBW).

HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).

PER = Protein efficiency ratio = ((FBW-initial body weight, IBW)/protein intake).

GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.

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Table 4. Effect of the experimental diets on digestive enzyme activities in European sea bass juveniles

	Experimental diets							
	Time 4				Time 8			
	FM20	FM10	FM10+B	<i>P</i>	FM20	FM10	FM10+B	<i>P</i>
Pepsin	0.66±0.21	0.67±0.34	0.60±0.04	0.926	0.46±0.20	0.73±0.15	0.60±0.21	0.135
Trypsin	0.48±0.05 ^B	0.32±0.0 ^B	0.33±0.12	0.045	0.26±0.04 ^A	0.19±0.08 ^A	0.23±0.10	0.331
Chymotrypsin	5.48±1.89	4.60±2.34	2.81±0.33	0.225	3.08±0.65	3.83±0.71	3.34±0.82	0.287
Amylase	1.96±0.29 ^B	1.28±0.56	1.62±0.64	0.220	0.80±0.15 ^A	0.82±0.14	0.96±0.17	0.293
Lipase	0.033±0.007	0.036±0.014	0.044±0.003 ^B	0.361	0.027±0.007	0.028±0.005	0.030±0.009 ^A	0.840

812 Data are given as the mean (n=4) ± SD. Enzyme activity of the different experimental groups after 64 days of feeding,
813 expressed in U g⁻¹ body weight. Pepsin, amylase and lipase = U g⁻¹ BW *10³.
814 Different upper-case letters indicate significant difference (Student's T-test, *P* ≤ 0.05) within the same diet at different
815 sampling time.
816 FM20 = 200g kg⁻¹ fishmeal (FM); FM10 = 100g kg⁻¹ FM; FM10+B = 100g kg⁻¹ FM + 30g kg⁻¹ buffer (B, CaCO₃)
817 Time 4 = 4 hours post meal, hpm; Time 8 = 8 hpm
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Table 5. Alpha-diversity metrics values of gut bacterial community of European sea bass juveniles fed different diets. Reported data are expressed as means \pm SD (n = 3)

Diet	Shannon	Faith's PD	Chao1	Species evenness	OTUs	OTU-assigned sequences	High quality sequences
FM20	4.9 \pm 0.1	26 \pm 3	466 \pm 91	0.82 \pm 0.003	406 \pm 70	10024 \pm 22	35921 \pm 8690
FM10+B	5.5 \pm 0.6	41 \pm 31	2167 \pm 3024	0.86 \pm 0.010	635 \pm 475	11086 \pm 81	48351 \pm 2682
FM10	5.9 \pm 0.2	44 \pm 30	2171 \pm 2878	0.94 \pm 0.006	701 \pm 436	11839 \pm 268	35768 \pm 3897

Data are given as mean \pm SD (n = 3)

OTU = operational taxonomic unit

FM20 = 200g kg⁻¹ fishmeal (FM); FM10 = 100g kg⁻¹ FM; FM10+B = 100g kg⁻¹ FM + 30g kg⁻¹ buffer (B, CaCO₃).

No significant difference among treatment were detected (Mann-Whitney-Wilcoxon test, $P \leq 0.05$)

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844 **Figure captions**

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846 **Figure 1.**

847 Gastrointestinal pH (mean, $n=9 \pm$ standard deviation, SD) measured in the stomach (A),
848 anterior intestine (B), mid-intestine (C) and hindgut (D) during 12 hours post prandial
849 cycle in European sea bass juveniles fed different diets. (FM20 —) = 200g kg^{-1}
850 fishmeal (FM); (FM10 - - -) = 100g kg^{-1} FM; (FM10+B) = 100g kg^{-1} FM +
851 30g kg^{-1} buffering additive (B, CaCO_3). Different lower-case letters (a b; a' b'; a''b'';
852 FM20, FM10, FM10+B, respectively) indicate significant difference between sampling
853 time while different upper-case letters indicate significant difference between treatments
854 at each sample time (Two-way ANOVA, $P \leq 0.05$).

855 **Figure 2**

856 Relative abundance (%) of gut bacteria composition at phylum (A) and family levels (B)
857 measured in each tank in European sea bass juveniles fed different diets after 64 days. In
858 each tank analyses were performed on one pool of hindgut content originating from 3 fish
859 sampled per tank. FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B =
860 100g kg^{-1} FM + 30g kg^{-1} buffering additive (B, CaCO_3). Only taxa with relative
861 abundance $> 0.1\%$ in at least 1 sample were included.

862 **Figure 3**

863 Principal Coordinates Analysis (PCoA) plot based on the Bray-Curtis distances
864 representing the spatial distribution of the gut bacterial community belonging to the three
865 dietary treatments (FM20 – green; FM10+B - cyan and FM10 - red). FM20 = 200g kg^{-1}
866 fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffering
867 additive (B, CaCO_3). FM20 was significantly separated both from diet FM10+B (p value

868 0.017) and FM10 (p value 0.005). Groups FM10+B and FM10 showed a non-significant
869 separation (p value 0.09).

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871 **Figure 4**

872 Relative abundance (> 1%) of the most represented gut bacteria genera belonging to

873 Lactic acid bacteria (LAB) found in European sea bass juveniles fed different diets. FM20

874 = 200g kg⁻¹ fishmeal (FM); FM10 = 100g kg⁻¹ FM; FM10+B = 100g kg⁻¹ FM + 30g kg⁻¹

875 buffering additive (B, CaCO₃).

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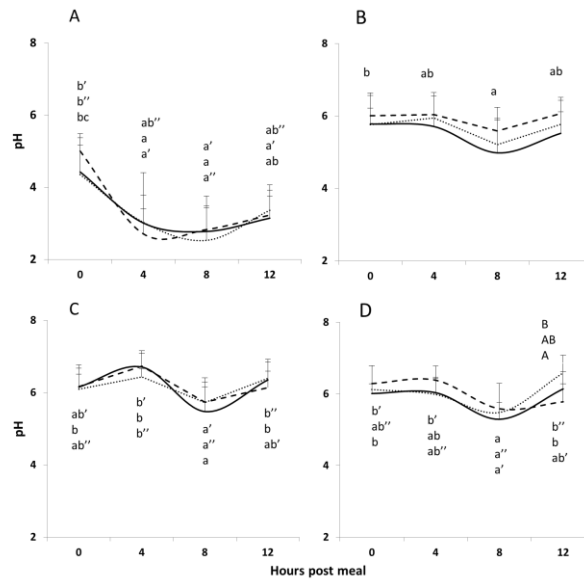
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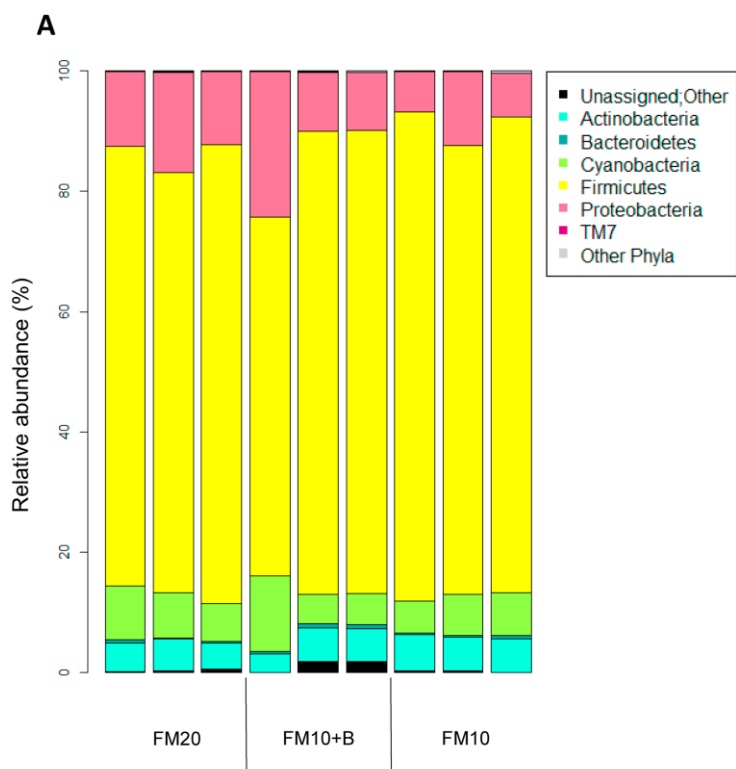
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883 Figure 1



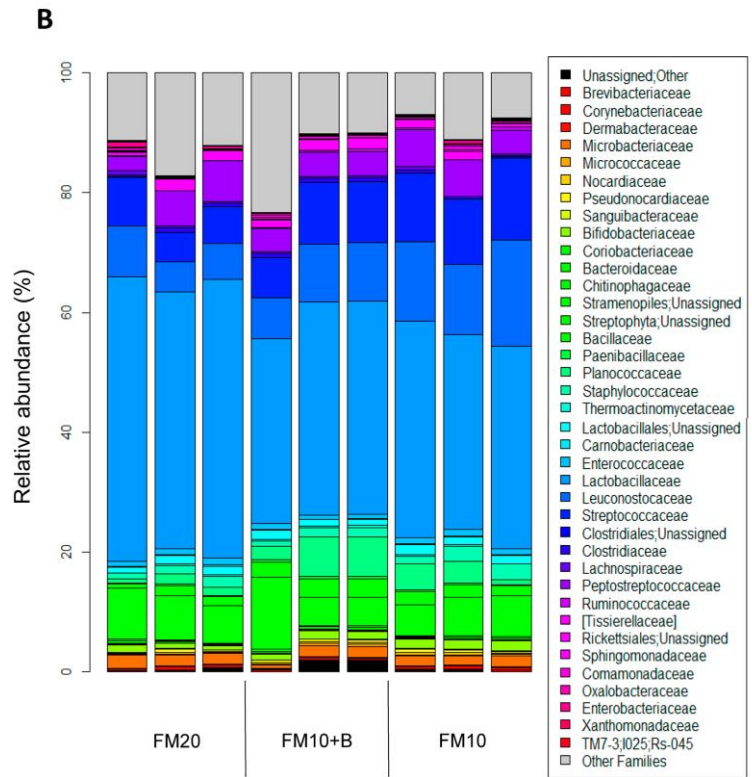
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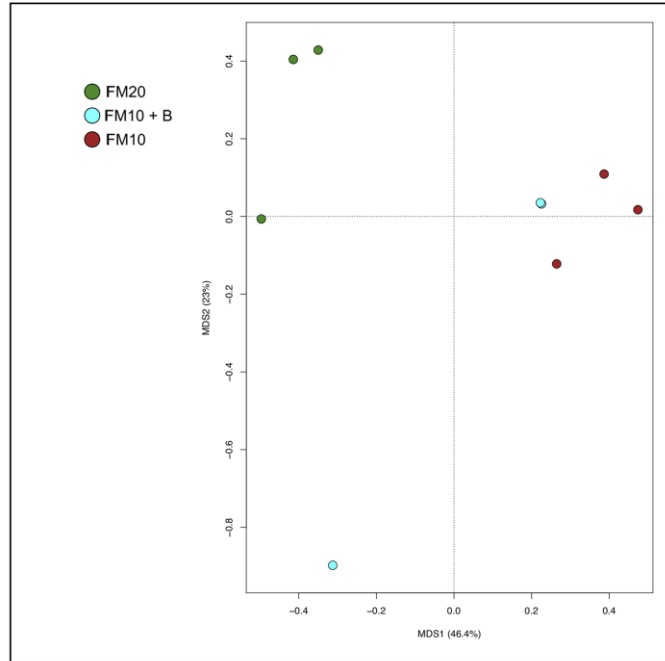
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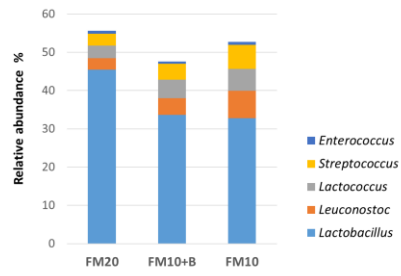
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950 Figure 4



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