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18 **Effects of rearing density on growth, digestive conditions, welfare indicators and gut**
19 **bacterial community of gilthead sea bream (*Sparus aurata*, L. 1758) fed different**
20 **fishmeal and fish oil dietary levels**

21

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40

41

42 **Abstract**

43

44 In Mediterranean aquaculture, significant advances have been made towards a
45 reduction of marine-derived ingredients in aquafeed formulation, as well as in defining
46 the effect on how environmental factors such as rearing density interact with fish health.
47 Little research, however, has examined the interaction between rearing density and
48 dietary composition on main key performance indicators, physiological processes and gut
49 bacterial community. A study was undertaken, therefore to assess growth response,
50 digestive enzyme activity, humoral immunity on skin mucus, plasma biochemistry and
51 gut microbiota of gilthead sea bream (*Sparus aurata*, L. 1758) reared at high (HD, 36-44
52 kg m⁻³) and low (LD, 12-15 kg m⁻³) final stocking densities and fed high (FM30/FO15,
53 30% fishmeal FM, 15% fish oil, FO) and low (FM10/FO3; 10% FM and 3% FO) FM and
54 FO levels. Isonitrogenous and isolipidic extruded diets were fed to triplicate fish groups
55 (initial weight: 96.2 g) to overfeeding over 98 days. The densities tested had no major
56 effects on overall growth and feed efficiency of sea bream reared at high or low FM and
57 FO dietary level. However, HD seems to reduce feed intake compared to LD mainly in
58 fish fed FM30/FO15. Results of digestive enzyme activity indicated a comparable
59 digestive efficiency among rearing densities and within each dietary treatment even if
60 intestinal brush border enzymes appeared to be more influenced by stocking density
61 compared to gastric and pancreatic enzymes. Plasma parameters related to nutritional and
62 physiological conditions were not affected by rearing densities under both nutritional
63 conditions a similar observation was also achieved through the study of lysozyme,
64 protease, antiprotease and total protein determination in skin mucus, however; in this case
65 lysozyme was slightly reduced at HD. For the first time on this species, the effect of

66 rearing density on gut bacterial community was studied. Different response in relation to
67 dietary treatment under HD and LD were detected. Low FM-FO diet maintained steady
68 the biodiversity of the gut bacterial community between LD and HD conditions while fish
69 fed high FM-FO level showed a reduced biodiversity at HD. According to the results, it
70 seems feasible to rear gilthead sea bream at the on-growing phase at a density up to 36-
71 44 kg m⁻³ with low or high FM-FO diet without negatively affecting growth, feed
72 efficiency, welfare condition and gut bacterial community.

73

74 **Keywords**

75

76 Gilthead sea bream, rearing density, fishmeal and fish oil replacement, digestive
77 enzyme, humoral immunity on skin mucus, gut bacterial community.

78

79 **Introduction**

80

81 Despite the considerable advances addressing the study of nutritional requirements and
82 sustainable feed ingredients in fish, which have resulted in a deep knowledge about the
83 optimal composition of aquafeeds for Mediterranean fish species, technical performance
84 indicators such as growth, feed utilization and survival in Mediterranean aquaculture have
85 not improved over the last decade. The intensification of production systems and their
86 possible effects on stress and welfare or the less explored interaction between nutrition,
87 feeding management and suboptimal environmental conditions may have contributed to
88 this stagnation. Among stress factors, inadequate rearing density has been recognized as
89 a source of chronic stress in fish species which could affect physiological processes such

90 as osmoregulation or immune competence, mobilization of energy sources and alterations
91 in behaviour, which are generally translated into a decreased feed intake, reduced feed
92 efficiency and decreased growth performance (Ellis et al., 2002; Tort et al., 2011). In
93 gilthead sea bream (*Sparus aurata*), several studies have evaluated the effects of stocking
94 density on growth and fish health. In juveniles, Canario et al. (1998) found that growth
95 was negatively correlated to stocking density when fish were reared at a final stocking
96 density of 16.8 kg m⁻³ compared to 2.4 kg m⁻³, while Montero et al. (1999) did not find
97 an effect on growth and feed intake when specimens (22-85 g) were reared up to 40.8 kg
98 m⁻³, even if a negative effect on plasma and serum parameters were detected. More
99 recently high stocking density (final density 57 kg m⁻³) decreased growth performance,
100 feed intake and feed efficiency of gilthead sea bream (12-58 g) in comparison to lower
101 density 5-26 kg m⁻³ (Diógenes et al., 2019). In addition, in adult fish (272-425g) rearing
102 density was increased up to 20 kg m⁻³ without affecting physiological parameters and
103 growth, when oxygen level was maintained above 70% of the saturation level (Araujo-
104 Luna et al., 2018). Concerning the effect of rearing density on welfare in this species,
105 several studies have elucidated the effect on different physiological parameters, including
106 plasma parameters, neuroendocrine factors, skin mucus biomarkers, liver proteome,
107 carbohydrate metabolism of several tissues and behavioural studies (Montero et al., 1999;
108 Sangiao-Alvarellos et al., 2005; Mancera et al., 2008; Alves et al., 2010; Sánchez-Muros
109 et al., 2017; Guardiola et al., 2018; Skrzynska et al., 2018; Diógenes et al., 2019). Most
110 of those studies were conducted using standard diets and whether these density-associated
111 changes in performance and welfare are consistent when fish are fed current low fishmeal
112 (FM) and fish oil (FO) diets remains little investigated (Wong et al., 2013). In addition,
113 only a few studies in fish species have evaluated whether the interaction between stocking

114 density and diet composition may affect gut microbiota and none of these have been
115 evaluated in gilthead sea bream. The exposure to stress factors can impact the gut
116 microbiome community profile by altering the relative proportions of the main microbiota
117 phyla (Galley et al., 2014), while a recent study on blunt snout bream (*Megalobrama*
118 *amblycephala*) provided new evidence that the gut microbiome might be involved in the
119 response to crowding and consequently to the adaptation of fish to environmental
120 stressors (Du et al., 2019). The aim of the present study was to explore the effect of high
121 and low rearing density on growth, digestive enzyme activity, plasma biochemistry,
122 humoral immunity of skin mucus and gut microbiome structure during the on-growing of
123 gilthead sea bream fed low and high FM and FO dietary levels.

124

125 **Materials and methods**

126

127 *2.1 Experimental diets*

128

129 Ingredients and proximate composition of the experimental diets are presented in
130 Table 1. Two isonitrogenous (46% protein) and isolipidic (17% lipid) diets were
131 formulated to contain high and low FM and FO dietary levels (FM30/FO15 and
132 FM10/FO3; 30% FM, 15% FO and 10% FM and 3% FO, respectively). Diets were
133 formulated with FM and with a mixture of vegetable ingredients currently used for sea
134 bream in aquafeed (Parma et al., 2016). The diets were produced via extrusion (pellet size
135 = 4.0 mm) by SPAROS Lda (Portugal).

136

137 *2.2 Fish density and rearing*

138

139 The experiment was carried out at the Laboratory of Aquaculture, Department of
140 Veterinary Medical Sciences of the University of Bologna (Cesenatico, Italy). Gilthead
141 sea bream were obtained from the fish farm Cosa s.r.l (Orbello, GR) and adapted to the
142 laboratory facilities for 10 days before the beginning of the trial. Afterwards, two rearing
143 densities (low density and high density, LD and HD, respectively) were established by
144 randomly distributing 40 and 120 fish per tank ($96.2 \pm 2.1\text{g}$) in six 800L tanks
145 corresponding to an initial density of 4.8 and 14.4 kg m^{-3} , respectively (Table 2).

146 Each diet was administered to triplicate tanks at both rearing densities over 98 days.
147 Tanks were provided with natural seawater and connected to a closed recirculation system
148 (overall water volume: 15 m^{-3}). The rearing system consisted of a mechanical sand filter
149 (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25mJ cm^{-2} : $32 \text{ m}^{-3} \text{ h}^{-1}$,
150 Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain).
151 The water exchange rate within each tank was 100% every hour, while the overall water
152 renewal amount in the system was 5% daily. During the trial, the temperature was kept at
153 $24 \pm 1.0 \text{ }^\circ\text{C}$ and the photoperiod was maintained at 12 h light and 12 h dark by means of
154 artificial light. The oxygen level was kept constant ($8.0 \pm 1.0 \text{ mg L}^{-1}$) through a liquid
155 oxygen system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy).
156 Ammonia (total ammonia nitrogen $\leq 0.1 \text{ mg L}^{-1}$) and nitrite ($\leq 0.2 \text{ mg L}^{-1}$) were daily
157 monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business,
158 Darmstadt, Germany) while salinity (30 g L^{-1}) was measured by a salt refractometer (106
159 ATC). Sodium bicarbonate was added on a daily basis to keep pH constant at 7.8–8.0.
160 Fish were fed *ad libitum* twice a day (8:30, 16:30) for six days a week (one meal on
161 Sundays) via automatic feeders using an overfeeding approach with a daily feeding ration

162 10% higher than the daily ingested ration of the previous days as reported by Bonvini et
163 al. (2018a). Each meal lasted 1 h, after which the uneaten pellets of each tank were
164 collected, dried overnight at 105°C, and weighted for overall calculation.

165

166 *2.3 Sampling*

167

168 At the beginning and at the end of the experiment, all the fish in each tank were
169 anaesthetised by 2-phenoxyethanol at 300 mg L⁻¹ and individually weighed. The
170 proximate composition of the carcasses was determined at the beginning of the trial on a
171 pooled sample of 10 fish and on a pooled sample of 5 fish per tank at the end of the trial.

172 At the end of the trial, for the assessment of the specific activity of gastric (pepsin) and
173 pancreatic (trypsin, chymotrypsin, total alkaline proteases, α -amylase and bile salt-
174 activated lipase) digestive enzymes, 3 fish per tank (n = 9 fish per diet treatment) at 5
175 hours post meal (hpm) were randomly sampled, euthanized with overdose anaesthetic and
176 immediately eviscerated. The alimentary tract was dissected, adherent adipose and
177 connective tissues carefully removed and the gastrointestinal tract was stored at -80 °C
178 until their analysis. For the analysis of intestinal enzymes (alkaline phosphatase, maltase,
179 aminopeptidase-N and leucine-alanine peptidase), 3 fish per tank were sampled at 8 hpm,
180 at the same time, after fish dissection, anterior and posterior intestines were dissected and
181 stored at -80 °C until their analysis. Sampling times were selected in order to maximize
182 pancreatic enzyme levels in the stomach and anterior region of the intestine coinciding
183 with their maximal secretion into the gut from the exocrine pancreas due to the presence
184 of feed in the gut, while the activity of intestinal enzymes was measured at the end of the
185 digestion process (Deguara et al., 2013). The measurements of digestive enzymes was

186 then obtained by pooling the 3 fish sampled per tank during the analyses, as the tank was
187 considered as the experimental unit and not the organism. At the same time, digesta
188 content from posterior intestine (n = 15 fish per diet treatment, n = 5 fish per replicate)
189 was also individually sampled and immediately stored at -80 °C for gut microbiota
190 analysis according to Parma et al. (2016).

191 For the assessment of plasma biochemistry, blood from 5 fish per tank (n=15 fish per
192 diet treatment) was collected from the caudal vein. Samples were then centrifuged (3000
193 x g, 10 min, 4°C) and plasma aliquots were stored at -80 °C until analysis (Bonvini et al.,
194 2018b). Skin mucus samples were collected from 8 fish per tank according to the method
195 of Guardiola et al. (2014). Briefly, skin mucus was collected by gently scraping the
196 dorsolateral surface of specimens using a cell scraper, taking care to avoid contamination
197 with urino-genital and intestinal excretions. Collected mucus samples were then stored at
198 -80 °C until analyses.

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

202

203 *2.4 Calculations*

204

205 The following formulae were used to calculate different performance parameters:
206 specific growth rate (SGR) (% day⁻¹) = 100 * (ln FBW - ln IBW) / days (where FBW and
207 IBW represent the final and the initial body weights, respectively). Feed Intake (FI) (g kg
208 ABW⁻¹ day⁻¹) = ((1000 * total ingestion) / (ABW)) / days (where average body weight,
209 ABW = (IBW + FBW) / 2. Feed conversion ratio (FCR) = feed intake / weight gain. Protein

210 efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE)
211 (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total
212 protein intake fish. Gross lipid efficiency (GLE) = 100 * [(final body lipid (%) * FBW) -
213 (initial body lipid (%) *IBW)] / total lipid intake fish. Lipid efficiency ratio (LER) =
214 [(FBW-IBW)/lipid intake].

215

216 *2.5 Proximate composition analysis*

217

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

226

227 *2.6 Digestive enzyme activity*

228

229 Determination of pancreatic (α -amylase, bile salt-activated lipase, total alkaline
230 proteases), gastric (pepsin) and intestinal (alkaline phosphatase, aminopeptidase-N,
231 maltase and leucine-alanine peptidase) digestive enzymes were based on methods
232 previously described by Gisbert et al. (2009). In addition, spectrophotometric analyses
233 were performed as recommended by Solovyev and Gisbert (2016) in order to prevent

234 sample deterioration. In brief, the stomach and pyloric caeca samples (including 1 cm of
235 anterior intestine) were homogenized in 5 volumes (ww/v) of distilled water at 4 °C for
236 1 min followed by a sonication process of 30 sec. After a centrifugation (9,000 x g for 10
237 min at 4 °C), the supernatant was collected, aliquoted and stored at -20°C for the
238 quantification of gastric and pancreatic digestive enzymes.

239 Regarding intestinal enzymes, the anterior and posterior intestine samples were
240 homogenized in 30 volumes (w/v) of ice-cold Mannitol (50 mM), Tris-HCl buffer (2 mM)
241 pH 7.0, at a maximum speed for 30 s (IKA, Ultra-turrax[®], USA), then 100 µL of 0.1M
242 CaCl₂ was added to the homogenate, stirred and centrifuged (9,000 x g for 10 min at 4
243 °C). A fraction of the supernatant was collected and stored at -20 °C for the leucine-
244 alanine peptidase (LAP) activity quantification. After a second centrifugation (3,400 x g
245 for 20 min at 4 °C), the supernatant was discarded, and the pellet containing the intestinal
246 brush border enzymes (alkaline phosphatase, aminopeptidase-N and maltase) dissolved
247 in 1 mL of Tris-Mannitol.

248 Total alkaline protease activity was measured using azocasein (0.5%) as substrate in
249 Tris-HCl 50 nmol L⁻¹ (pH = 9). One unit (U) of activity was defined as the nmoles of azo
250 dye released per minute and per mL of tissue homogenate, and the absorbance read at λ
251 = 366 nm. Trypsin activity was assayed using BAPNA (N-α-benzoyl-DL-arginine p-
252 nitroanilide) as substrate. One unit of trypsin per mL (U) was defined as 1 µmol BAPNA
253 hydrolyzed min⁻¹ mL⁻¹ of enzyme extract at λ = 407 nm (Holm et al., 1988).
254 Chymotrypsin activity was quantified using BTEE (benzoyl tyrosine ethyl ester) as
255 substrate and its activity (U) corresponded to the µmol BTEE hydrolyzed min⁻¹ mL⁻¹ of
256 enzyme extract at λ = 256 nm (Worthington, 1991). Alpha-amylase activity was
257 determined using 0.3% soluble starch as substrate (Métais and Bieth, 1968), and its

258 activity (U) was defined as the amount of starch (mg) hydrolysed during 30 min per mL
259 of tissue homogenate at $\lambda = 580$ nm. Bile salt-activated lipase activity was assayed for 30
260 min using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture
261 of acetone: n-heptane (5:2), the extract centrifuged (2 min at $6,080 \times g$ and 4°C) and the
262 increase in absorbance of the supernatant read at $\lambda = 405$ nm. Lipase activity (U) was
263 defined as the amount (nmol) of substrate hydrolyzed per min per mL of enzyme extract
264 (Iijima et al., 1998). Pepsin activity (U) was defined as the nmol of tyrosine liberated per
265 min per mL of tissue homogenate read at $\lambda = 280$ nm (Worthington, 1991).

266 Regarding intestinal digestive enzymes, alkaline phosphatase was quantified using 4-
267 nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as $1 \mu\text{mol}$ of pNP
268 released $\text{min}^{-1} \text{mL}^{-1}$ of brush border homogenate at $\lambda = 407$ nm (Gisbert et al., 2018).
269 Aminopeptidase-N was determined using 80mM sodium phosphate buffer (pH = 7.0) and
270 L-leucine p-nitroanilide as substrate (in 0.1 mM DMSO) (Maroux et al., 1973). One unit
271 of enzyme activity (U) was defined as $1 \mu\text{g}$ nitroanilide released per min per mL of brush
272 border homogenate at $\lambda = 410$ nm. Maltase activity was determined using d(+)-maltose
273 as substrate in 100 mM sodium maleate buffer (pH = 6.0) (Dahkqvist, 1970). One unit of
274 maltase (U) was defined as μmol of glucose liberated per min per mL of homogenate at
275 $\lambda = 420$ nm. The assay of the cytosolic peptidase, LAP was performed on intestinal
276 homogenates applying the method described by Nicholson and Kim (1975) which utilized
277 L-alanine as substrate in 50 mM Tris-HCl buffer (pH = 8.0). One unit of enzyme activity
278 (U) was defined as 1 nmol of the hydrolyzed substrate $\text{min}^{-1} \text{mL}^{-1}$ of tissue homogenate
279 at $\lambda = 530$ nm. Soluble protein of crude enzyme extracts was quantified by means of the
280 Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All
281 enzymatic activities were measured at $25\text{-}26^\circ\text{C}$ and expressed as specific activity defined

282 as units per mg of protein (U mg protein^{-1}). All the assays were made in triplicate
283 (methodological replicates) for each tank and the absorbance was read using a
284 spectrophotometer (TecanTM Infinite M200, Switzerland).

285

286 *2.7 Humoral immunity on skin mucus*

287 *2.7.1. Lysozyme, protease, antiprotease and total protein determination*

288

289 Lysozyme activity was measured according to the turbidimetric method described by
290 Swain et al. (2007). Briefly, 20 μL of skin mucus were placed in flat-bottomed 96-well
291 plates. To each well, 180 μL of freeze-dried *Micrococcus lysodeikticus* (0.2 mg mL^{-1} ,
292 Sigma-Aldrich) in 40 mM sodium phosphate (pH 6.2) was added as lysozyme substrate.
293 As blanks of each sample, 20 μL of skin mucus were added to 180 μL of sodium
294 phosphate buffer. The absorbance at $\lambda = 450 \text{ nm}$ was measured after 20 min at 35 °C in a
295 microplate reader (BMG Labtech). The amounts of lysozyme present in the samples were
296 obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma)
297 through serial dilutions in the above buffer. Skin mucus lysozyme values are expressed
298 as U mL^{-1} equivalent of HEWL activity.

299 Protease activity was quantified using the azocasein hydrolysis assay according to
300 Guardiola et al. (2014). Aliquots of 100 μL of each mucus sample were incubated with
301 100 μL of 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma-
302 Aldrich) for 19 h at 30 °C. The reaction was stopped by adding 4.6% trichloro acetic acid
303 (TCA) and the mixture centrifuged ($10,000 \times g$, 10 min). The supernatants were
304 transferred to a 96-well plate in triplicate containing 100 $\mu\text{L well}^{-1}$ of 0.5 N NaOH. In
305 both cases, the OD was read at $\lambda = 450 \text{ nm}$ using a plate reader. Skin mucus was replaced

306 by trypsin (5 mg mL⁻¹, Sigma), as positive control (100% of protease activity), or by
307 buffer, as negative controls (0 % of protease activity).

308 Total antiprotease activity was determined in skin mucus by its ability to inhibit trypsin
309 activity (Hanif et al., 2004). Briefly, 10 µL of skin mucus were incubated (10 min, 22 °C)
310 with the same volume of standard trypsin solution (5 mg mL⁻¹) in a 96-well flat-bottomed
311 plate. After adding a volume of 100 µL of 100 mM ammonium bicarbonate buffer and
312 125 µL of buffer containing 2% azocasein (Sigma), samples were incubated (2 h, 30 °C)
313 and, following the addition of 250 µL 10% TCA, were incubated again (30 min, 30 °C).
314 The mixture was then centrifuged (10,000 x g, 10 min) and the supernatant was
315 transferred to a 96-well plate in triplicate, containing 100 µL well⁻¹ of 1 N NaOH before
316 the OD was read at $\lambda = 450$ nm using a plate reader. For a positive control, the reaction
317 buffer replaced mucus and trypsin, and for a negative control, the reaction buffer replaced
318 the mucus. The antiprotease activity was expressed in terms of the percentage of trypsin
319 inhibition according to the formula: % Trypsin inhibition = (Trypsin OD – Sample OD)/
320 Trypsin OD x 100.

321 Skin mucus protein concentration was determined by the dye binding method of
322 Bradford (1976) using bovine serum albumin (BSA, Sigma-Aldrich) as the standard.
323 Briefly, 2 mg mL⁻¹ solution of BSA was prepared and serial dilutions made with
324 phosphate buffer saline (PBS Sigma-Aldrich) as standards. Dilutions of 5 µL of skin
325 mucus and 15 µL of PBS were prepared. Then 250 µL of Bradford reagent (Sigma-
326 Aldrich) was added to BSA and skin mucus dilutions and incubated at room temperature
327 for 10 min. The absorbance of each sample was then read at $\lambda = 595$ nm and the results
328 were taken and plotted onto the standard curve to obtain the total protein content of skin

329 mucus. All spectrophotometry reads were conducted with a Varioskan 2.4.5, (Thermo
330 Scientific, MA, USA).

331

332 *2.8 Gut bacterial community DNA extraction and sequencing*

333

334 Total bacterial DNA was extracted and analysed from individual distal intestine
335 content obtained from 5 fish per tank as previously reported in Parma et al. (2019).
336 Afterwards, the V3–V4 hypervariable region of the 16S rRNA gene was amplified using
337 the 341F and 785R primers (Klindworth et al., 2013) with added Illumina adapter
338 overhang sequences and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems).
339 Briefly, the thermal cycle consisted of an initial denaturation at 95 °C for 3 min, 30 cycles
340 of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72°C for
341 30 s, and a final extension step at 72 °C for 5 min. PCR reactions were cleaned up for
342 sequencing by using Agencourt AMPure XP magnetic beads as recommended in the
343 Illumina protocol “16S Metagenomic Sequencing Library Preparation” for the MiSeq
344 system, and as used in several other publications (Biagi et al., 2018; Soverini et al., 2016).
345 Sequencing was performed on Illumina MiSeq platform using a 2 x 250 bp paired-end
346 protocol according to the manufacturer’s instructions (Illumina, San Diego, CA). The
347 sequencing process resulted in a total of 1,553,593 high quality reads that were processed
348 using the QIIME 2 pipeline (Bolyen et al., 2019). After length (minimum/maximum =
349 250/550 bp) and quality filtering with default parameters, reads were cleaned using
350 DADA2 (Callahan et al., 2016) and clustered into OTUs at a 0.99 similarity threshold
351 using VSEARCH (Rognes et al., 2016). Assignment was carried out by using the RDP
352 classifier against Silva database (Quast et al., 2013).

353

354 *2.9 Metabolic parameters in plasma*

355

356 The levels of glucose (GLU), urea, creatine, uric acid, total bilirubin, bile acid,
357 amylase, lipase, cholesterol (CHOL), triglycerides (TRIG), total protein (TP), albumin
358 (ALB), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline
359 phosphatase (ALP), gamma-glutamyl transferase (GGT), creatine kinase (CK), lactate
360 dehydrogenase (LDH), calcium (Ca^{+2}), phosphorus (P), potassium (K^+) sodium (Na^+),
361 iron (Fe), chloride (Cl), magnesium (Mg), unsaturated iron binding capacity (UIBC), total
362 iron binding capacity (TIBC) and cortisol were determined in the plasma using samples
363 of 500 μL on an automated analyser (AU 400; Beckman Coulter) according to the
364 manufacturer's instructions. The ALB/globulin (GLOB), Na/K ratio and Ca x P were
365 calculated.

366

367 *2.10 Statistical analysis*

368

369 All data are presented as mean \pm standard deviation (SD). A tank was used as the
370 experimental unit for analysing growth performance and a pool of five and three sampled
371 fish were considered the experimental unit for analysing carcass composition and enzyme
372 activity respectively. Individual fish were used for analysing plasma biochemistry and
373 mucus stress parameters. Data of growth performance, nutritional indices, enzyme
374 activity, plasma and skin mucus parameters were analysed by a two-way analysis of
375 variance (ANOVA) and in case of significance ($p \leq 0.05$) Tukey's post hoc test was
376 performed. The normality and/or homogeneity of variance assumptions were validated

377 for all data preceding ANOVA. The R packages “Stats” and “Vegan” were used to
378 perform gut microbiota statistical analysis. In particular, to compare the microbiota
379 structure among different groups for alpha and beta-diversity, Wilcoxon rank-sum test
380 was used while the PCoA was tested using a permutation test with pseudo-F ratios
381 (function “Adonis” in the “Vegan” package). Alpha diversity of the different ecosystems
382 was computed using Hill numbers (Hill, 1973; Chao et al., 2014). Beta diversity was
383 estimated using both weighted and unweighted UniFrac metrics. Statistical analyses were
384 performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA,
385 USA) and RStudio interface for R (<https://www.r-project.org>). The differences among
386 treatments were considered significant at $p \leq 0.05$.

387

388 **3. Results**

389

390 *3.1 Growth*

391

392 Results on growth performance parameters are summarised in Table 2. No significant
393 effects on growth (FBW, weight gain and SGR) were detected between LD and HD
394 groups for both dietary treatments ($p > 0.05$). However, fish fed FM30/FO15 displayed
395 higher FBW, weight gain and SGR values compared to the FM10/FO3 group ($p < 0.05$).
396 Values of FI were lower in HD compared to LD (density effect $p = 0.002$) with more
397 marked differences in FM30/FO15 than FM10/FO3, whereas no significant diet effect on
398 FI was detected ($p > 0.05$). No significant effect of density on FCR was observed ($p >$
399 0.05), while the FM10/FO3 group showed higher FCR values, followed by FM30/FO15.
400 Survival rates were lower in the LD group ($p < 0.05$).

401 Data on body composition and nutritional indices are shown in Table 3. Whole body
402 composition values were not significantly influenced by different fish density ($p > 0.05$),
403 while lipid content was lower in fish fed the FM10/FO3 diet compared to the FM30/FO15
404 group ($p < 0.05$); however, ash and moisture levels were higher in FM10/FO3 than
405 FM30/FO15 fish ($p < 0.05$). No significant effects of fish density on PER, GPE, GLE and
406 LER were detected ($p > 0.05$); however, fish fed FM10/FO3 displayed lower PER, GPE,
407 GLE and LER compared to FM30/FO15 ($p < 0.05$).

408

409 *3.2 Digestive enzyme activity*

410

411 Data on specific activity of gastric, pancreatic and intestinal digestive enzymes are
412 shown in Table 4. The activities of both pancreatic (trypsin, chymotrypsin, total alkaline
413 proteases, amylase and bile salt-activated lipase) and gastric (pepsin) enzymes were not
414 significantly affected by the rearing density nor the diet ($p > 0.05$); with the exception of
415 trypsin, which was slightly affected by the diet composition ($p = 0.053$) with lower values
416 recorded in fish fed the FM10/FO3 diet compared to those fed the FM30/FO15 diet.
417 Regarding intestinal brush border enzymes measured in the anterior segment of the
418 intestine, aminopeptidase-N and maltase activities were not significantly affected by the
419 diet nor rearing density ($p > 0.05$), while phosphatase alkaline and LAP were slightly (p
420 < 0.1) lower in FM10/FO3 than FM30/FO15. The activity of LAP was significantly
421 higher at HD compared to LD for both dietary treatments ($p < 0.05$). Concerning the
422 intestinal enzymes measured in the posterior region of the intestine, aminopeptidase and
423 LAP were significantly affected by the rearing density with lower values recorded at HD
424 in comparison to those recorded in fish kept at LD ($p < 0.05$). Diet significantly affected

425 aminopeptidase-N and maltase activities which were significantly lower in sea bream fed
426 the FM10/FO3 diet ($p < 0.05$). No significant effects of both diets and tested densities
427 were detected in the phosphatase alkaline activities in the posterior intestine ($p > 0.05$).

428

429 *3.3 Plasma biochemistry*

430

431 The results of plasma parameters are shown in Table 5. No significant effect ($p > 0.05$)
432 of density on plasma parameters was detected under both feeding regimes. Concerning
433 the effect of diet on plasmatic parameters like urea, lipase, UIBC, A/G, TIBC, Na^+ , K^+ ,
434 Cl^- , these were higher in fish from the FM10/FO3 group compared to those from the
435 FM30/FO15 group ($p < 0.05$), while creatine, Ca^{2+} , Mg, CHOL, TP, ALB and Na^+/K^+
436 were lower in FM10/FO3 compared to FM30/FO15 fish ($p < 0.05$). No significant
437 differences related to density and feeding regimes for GLU, uric acid, creatine, total
438 bilirubin, AST, ALT, ALP, amylase, GGT, CK, LDH, P, TRIG, Bile acid, CaxP, Fe and
439 cortisol were detected among experimental groups ($p > 0.05$).

440

441 *3.4 Skin mucus non-specific immune biomarkers*

442

443 Results of skin mucus lysozyme, protease, antiprotease and total proteins are presented
444 in Figure 1 (A-D). Lysozyme activity was slightly affected by the rearing density (density
445 effect $p = 0.04$) with higher values recorded under LD rearing conditions. Specifically,
446 lysozyme was significantly higher in fish fed FM30/FO15 at LD rearing conditions
447 compared to those fed FM10/FO3 and reared at HD (Fig 1A; $p < 0.05$). Protease was
448 significantly reduced under fish fed FM10/FO3 (diet effect $p = 0.0006$), while no

449 significant effect of rearing density was detected ($p > 0.05$). Specifically, protease activity
450 in skin mucus was significantly higher in fish fed the FM30/FO15 diet at both rearing
451 densities compared to those fed FM10/FO3 and reared at LD (Fig 1B; $p < 0.05$). No
452 significant effect of density or diet were detected in antiprotease activity and total proteins
453 of skin mucus from fish belonging to the different experimental groups (Fig. 1, C-D; $p >$
454 0.05).

455

456 3.5 Gut bacterial community profiles

457

458 Taxonomic characterisation of the gut bacterial community at different phylogenetic
459 levels is represented in Figure 2: phylum in panel (A) and family in panel (B) and in
460 Supplementary Table 1. At phylum level, the most abundant taxa were Firmicutes,
461 Actinobacteria and Proteobacteria. In addition, the families most represented, all
462 belonging to Firmicutes phylum, were *Lactobacillaceae* (FM30/FO15_{HD}: 77.9% \pm
463 16.1%; FM30/FO15_{LD}: 86.5% \pm 4.4%; FM10/FO3_{HD}: 61.3% \pm 12.4%; FM10/FO3_{LD}:
464 67.6% \pm 12.2%), *Streptococcaceae* (FM30/FO15_{HD}: 2.0% \pm 1.5%; FM30/FO15_{LD}: 1.3%
465 \pm 1.4%; FM10/FO3_{HD}: 4.1 % \pm 3.7%; FM10/FO3_{LD}: 3.2% \pm 2.3%) and
466 *Staphylococcaceae* (FM30/FO15_{HD}: 1.4 % \pm 1.0 %; FM30/FO15_{LD}: 0.9 % \pm 0.4 %;
467 FM10/FO3_{HD}: 0.6% \pm 1.3%; FM10/FO3_{LD}: 0.3% \pm 0.5%). No significant differences
468 (Wilcoxon test $p > 0.05$, FDR correction) among groups at phylum level were detected
469 between specimens fed with the same diet but in different rearing density condition. On
470 the other hand, significant differences in several families such as *Staphylococcaceae* were
471 observed, values that were higher in the FM30/FO15_{HD} group than in FM10/FO3_{HD} group
472 ($p < 0.05$, Wilcoxon rank-sum test), and *Streptococcaceae*, higher in FM10/FO3_{HD} group

473 compared to FM30/FO15_{HD} group ($p < 0.05$). Moreover, at LD, both diets determined a
474 significant difference in the abundance of *Lactobacillaceae* and *Staphylococcaceae*, both
475 higher in FM30/FO15_{LD} group compared to FM10/FO3_{LD} ($p < 0.05$, Wilcoxon rank-sum
476 test) (Figure 2 C).

477 The biodiversity among microbiota from fish fed different diets and kept at different
478 stocking densities, expressed using Hill numbers of different magnitudes (from $q = 0$ to
479 $q = 2$), is represented in panel A of Figure 3. For all the q value magnitude, diet FM10/FO3
480 is characterised by a more even distribution of bacterial species characteristic that is
481 strengthened going from order $q = 0$ to order $q = 2$. According to the results, diet FM10/FO3
482 was more effective in the maintenance of a greater biodiversity in the sea bream gut
483 ecosystem. Furthermore, it is interesting to notice that for a $q = 0$, diet FM30/FO15
484 showed a number of species comparable to diet FM10/FO3, shifting to a significantly
485 more uneven ecosystem ($p < 0.05$, t-test) increasing the weight of the microbial core (q
486 values of 1 and 2, respectively). These results also showed that the response to rearing
487 conditions shifted depending on the fishes feeding regimen: diet FM10/FO3 maintained
488 steady the biodiversity of the gut microbiota between HD and LD (p value > 0.05 ; t-test).
489 On the other hand, diet FM30/FO15 was not able to maintain the evenness of the
490 community, as highlighted in the q value of 2, in which the FM30/FO15_{HD} group showed
491 a significantly reduced biodiversity when compared to the other groups (p value < 0.05 ,
492 t-test). To assess whether these different treatments could influence the gut bacterial
493 ecosystem, a multivariate analysis was performed. In both Principal Coordinates Analysis
494 (PCoA) graphs obtained using both weighted UniFrac metric (Figure 3 B) and
495 unweighted UniFrac metric (Figure 3 C) a significant separation was observed between

496 the different groups in the two-dimensional space (Adonis $p < 0.01$), except for
497 FM30/FO15_{HD} vs FM30/FO15_{LD} which did not show a significant ($p > 0.05$) separation.

498

499 **Discussion**

500

501 Several studies have investigated the effect of high rearing density on growth,
502 physiological responses and health in gilthead sea bream; however, studies concerning
503 the possible interaction between rearing density and low FM FO-based diets have been
504 less explored. In the present study, fish reared at high density ($14.5\text{-}36/44 \text{ kg m}^{-3}$, initial
505 and final density, respectively) within each FM and FO dietary levels showed similar
506 performance in terms of growth and feed utilisation in comparison to those reared at low
507 density ($4.8\text{-}12/15 \text{ kg m}^{-3}$). The results of the present study during the on-growing phase
508 ($96\text{-}318\text{g}$) go beyond the maximum density tested ($20\text{-}31 \text{ kg m}^{-3}$) by Araújo-Luna et al.
509 (2018) for gilthead sea bream at similar size ($268\text{-}435 \text{ g}$). The authors did not find any
510 negative effects of high rearing density on SGR even if a significant linear relationship
511 between FCR and increasing stocking densities was observed. Indeed, the results of the
512 present study are consistent with a previous observation reported on juveniles ($22\text{-}85 \text{ g}$)
513 in which high density up to 40.8 kg m^{-3} did not negatively affect growth (Montero et al.,
514 1999). However, more recently, Diogenes et al. (2019) found that rearing density up to
515 57 kg m^{-3} impaired FI, growth and FCR in sea bream juveniles ($12\text{-}58\text{g}$). The authors
516 suggested that 40 kg m^{-3} could be near the maximum tolerable stocking density for
517 gilthead sea bream of the weight range tested. This seems in agreement also for the size
518 tested in the present study; even if high density had no negative effect on the overall
519 growth and feed utilisation, high density significantly ($p = 0.002$) reduced FI.

520 Interestingly, this effect was mainly reported in high FM and FO dietary level and this
521 could be a consequence of the higher final stocking density obtained under this treatment
522 (44 vs 36 kg m⁻³, FM30/FO15, FM10/FM3, respectively) or be due to the fact that density
523 could have increased feeding competition only in a potentially more palatable and
524 digestible diet. The differences observed in growth performance between diets were
525 mainly related to a lower feed utilisation occurring in FM10/FO3; however it should be
526 taken into account that the growth performance achieved in the present trial under both
527 diets is in line with those found in literature for similar dietary formulation and that the
528 sole comparison between the two diets was not the purpose of the present study.

529 Stress conditions can disrupt the endocrine system and affect some physiological
530 functions such as digestive capacity (Trenzado et al., 2018). Few studies have evaluated
531 the effect of stocking density with a dietary interaction on digestive enzyme activity at
532 the on-growing stage in fish species (Wong et al., 2013). In the present study rearing
533 density did not affect pancreatic digestive enzyme specific activities under both dietary
534 treatments. Similarly, protease, lipase and amylase activities were not affected by rearing
535 density in gilthead sea bream fed increasing dietary tryptophan level with alternative
536 vegetable protein sources (Diogenes et al., 2019) or in Nile tilapia (*Oreochromis*
537 *niloticus*) fed dietary live and heat-inactive baker's yeast in vegetable-meal based diet
538 (Ran et al., 2016). Contrarily, Trenzado et al. (2018) studying the interaction between
539 stocking density and dietary lipid content in rainbow trout (*Oncorhynchus mykiss*) found
540 that stocking density inhibited the adaptive response of lipase activity and enhanced the
541 protease activity inhibition due to higher dietary lipid content. Compared to the pancreatic
542 enzyme activity, in the present study, density seemed to slightly affect the proteolytic
543 enzyme activity measured in the intestinal brush border of enterocytes. In particular, LAP

544 activity measured in the brush border of the anterior intestine tended to increase at high
545 density while aminopeptidase and LAP activity in the posterior intestine was slightly
546 reduced at high density in particular in the low FM-FO diet. The alkaline phosphatase of
547 the intestinal brush border is used as a marker of intestinal integrity and among its
548 functions was found to keep gastrointestinal inflammation under control (Lalles et al.,
549 2019, Messina et al., 2019). In addition, Nile tilapia reared at higher density displayed
550 higher alkaline phosphatase activity, possibly in line with higher pathogenic stressors at
551 high rearing density (Ran et al., 2016). In the present study, the absence of differences in
552 the alkaline phosphatase activity suggested no major functional changes in the integrity
553 of the intestine under different rearing density in both dietary treatments. The evaluation
554 of several plasma biochemical parameters is considered a valuable approach for assessing
555 the suitability of feeding practices, metabolic disorders, rearing conditions and presence
556 of acute or chronic stressors (Peres et al., 2013; Guardiola et al., 2018). No significant
557 effect of stocking density on any of the twenty-seven different plasma parameters
558 measured was detected under both dietary treatments. It is commonly accepted that high
559 stocking density generally leads to increased plasma cortisol levels in different fish
560 species, enhancing metabolic rate and compromising energy availability for several
561 physiological processes such as growth (Ashley, 2007). However, an opposite cortisol
562 response to stocking density has been also observed in some fish species suggesting that
563 cortisol response to stocking density is species-dependent and related to the gregarious
564 behaviour of the species at a specific stage of life (De las Heras et al., 2015; Millán-
565 Cubillo et al., 2016). Previous study of juveniles and adult sea bream held at high stocking
566 density, giving rise to chronic stress, showed significantly higher levels of plasma cortisol
567 than those held at low density, suggesting the incapacity of this species to reach adaptation

568 under chronic high rearing density conditions (Montero et al., 1999; Sangia-Alvarellos et
569 al., 2005). In accordance, TP, CHOL, TRIG were also found to be reduced at high
570 stocking density as a consequence of increased energy demand under stressful conditions
571 and possibly mediated by increased plasma cortisol (Diogenes et al., 2019). As also
572 reported for Senegal sole (*Solea senegalensis*) by Azeredo et al. (2019) the fact that fish
573 held at high density did not show higher plasma cortisol than their low-density
574 counterparts might be related to negative feedback mechanisms established in the HPI
575 axis, as a strategy of chronically stressed animals to attenuate an exacerbated stress
576 response (Bonga, 1997; Mommsen et al., 1999). In addition, the absence of effects of
577 rearing density on GLU, CHOL, TP and TRIG, suggests that the differences in rearing
578 density were not able to alter the metabolic processes related to growth and feed
579 utilisation. Non-specific plasma enzymes, such as AST, GGT, ALP, CK and LDH are
580 considered useful indicators of the health status and their elevated plasma level may
581 indicate specific tissue damage of several organs including liver, muscle, spleen and
582 kidney related to pathological processes, toxic chemical exposure, or traumatic conditions
583 or hypoxia, whereas specific references for this species and age are few (Peres et al.,
584 2013; Guardiola et al., 2018). Values of AST, CK, GGT and LDH were found in the lower
585 part of the range proposed by Peres et al. (2013) for healthy juvenile sea bream (70 g) fed
586 FM-based diet at low rearing density (3-5 kg m⁻³) and in line with those found by
587 Guardiola et al. (2018) during a feeding trial in sea bream of similar size. Levels of ALP
588 were higher than values previously found by Peres et al. (2013) and Guardiola et al.
589 (2018), a difference which can be related to FI since this enzyme is involved in the
590 absorption and transport of lipid and carbohydrates from the intestine, and its intestinal
591 activities are positively correlated with food ingestion and growth rate (Lemieux et al.,

1999; Lalles et al., 2019). The values of plasma electrolytes provided in the trial were comparable with the values reported in sea bream (Peres et al., 2013; Guardiola et al., 2018) and sobaity sea bream (*Sparidentex hasta*) (Hekmatpoure et al., 2019). Plasma electrolytes are indicators of the secondary phase of stress response in fish, providing an indirect indication of altered plasma cortisol levels; in particular plasma phosphorus and calcium levels were found to be sensitive to fish stocking density (Hrubec et al., 2000) while potassium levels are accepted as a general indicator of stress in fish (Guardiola et al., 2018).

Evaluation of skin mucosal immunity has been proposed recently as a promising alternative stress assessment in fish species after stressful conditions including crowding or transportation, whereas data of specific mucosal component in response to different stressors are still scarce (Guardiola et al 2016; Sanahuja et al., 2019). Enzymes in the epidermal mucus such as lysozyme, protease and antiprotease play an important role in humoral and skin mucus defence acting directly on a pathogen, or activating and enhancing the production of various immunological components of fish subjected to stressful situations (Esteban, 2012; Guardiola et al., 2016). The present results indicate different effects of treatments on specific skin mucus components, lysozyme being slightly reduced by high rearing density while protease was mainly reduced by low FM-FO diets. Both enzymes have been shown to be modulated either by diet or environmental conditions in sea bream. Most studies have shown the possibility of increasing lysozyme activity of skin mucus by dietary additives, such as selenium nanoparticles, *Moringa oleifera* leaves or probiotics; but crowding conditions at 20 kg m⁻³ for 30 days has also been reported to lead to an increase in lysozyme gene expression in sea bream skin mucus (Cordero et al., 2016; Mansour et al., 2018; Dawood et al., 2019). Concerning protease

616 activity, Guardiola et al. (2016) found a significant increase in this activity after 24 and
617 48 h of acute 50 kg m⁻³ crowding stress. However, in the same study a reduction in the
618 protease activity was also found after 48 h. The effect of protease activity under chronic
619 stressful conditions has been poorly investigated. Easy et al. (2010) studied the skin
620 mucus components following short- and long-term handling stress in Atlantic salmon
621 (*Salmo salar*), and no correspondence between skin mucus component and plasma
622 cortisol level in long-term stress was observed, suggesting that the activation of mucus
623 proteases may have been triggered by short-term elevated cortisol levels or that skin
624 mucus protease activation could result from physical disturbances such as abrasion due
625 to netting or overcrowding. More studies are needed to understand the role played by skin
626 mucus on stress in fishes.

627 Although the study of the gut microbiota by next-generation sequencing (NGS) has
628 already been conducted in this species under different feeding treatment, no information
629 concerning the effects of rearing density on gut microbiota is available. According to our
630 findings, the gut bacterial community is dominated by Firmicutes (69.9-92.2%), followed
631 by Actinobacteria and Proteobacteria. The dominance of Firmicutes we observed is in
632 general agreement with the previous NGS-based survey of the gut bacterial community
633 in sea bream and other marine or freshwater species fed similar aquafeed ingredients
634 employed in the present study (FM, soy-derivates, corn glutens, wheat gluten and wheat
635 meal) (Parma et al., 2016, Rimoldi et al., 2018a, 2018b; Parma et al., 2019). However,
636 our data differ from previous findings concerning the gut bacterial community of gilthead
637 sea bream and other Mediterranean fish species which displayed a dominance of
638 Proteobacteria and detected Firmicutes as the subdominant component (Carda-Diéguez
639 et al., 2014; Gatesoupe et al., 2016, Piazzon et al., 2017). These works characterised the

640 mucosa-adherent gut microbiota, which could differ from the microbiota of the intestinal
641 lumen (Ringo et al., 2018). In this context, a recent comparison between mucosa-adherent
642 gut microbiota and intestinal lumen gut microbiota in sea bream highlighted the
643 dominance of Proteobacteria in the gut mucosa while Firmicutes dominated the intestinal
644 lumen in the same specimens (unpublished data). In addition, other studies revealed that
645 the differences in abundance between Firmicutes and Proteobacteria could also have been
646 related to the dietary composition. In rainbow trout, the presence of Proteobacteria was
647 favoured by an animal protein-based diet while the inclusion of at least 25% of plant
648 proteins in the diet favoured the presence of Firmicutes (Rimoldi et al., 2018b).

649 At the family level, the gut bacterial community of the present study was widely
650 dominated by *Lactobacillaceae* ranging from 61.3 to 86.5 %. The presence and the role
651 of *Lactobacillaceae* and other lactic acid bacteria (LAB) in fish species is still
652 controversial (Ringo et al., 2018). Several studies have associated a high LAB abundance
653 with a high inclusion level of dietary plant ingredients or functional additives in sea bream
654 (Parma et al., 2016; Rimoldi et al., 2018a) or other marine fish species (Apper et al., 2016;
655 Rimoldi et al., 2018b; Parma et al., 2019). However, some studies found a reduction in
656 LAB relative abundance when high FM replacement was also associated with a decrease
657 in performance (Estruch et al., 2015; Miao et al., 2018), while others found a higher
658 abundance of LAB in relation to vegetable protein associated with impaired gut health
659 (Gajardo et al., 2017). The results of the present study reinforce previous observation that
660 the dominance of *Lactobacillaceae* mainly *Lactobacillus* could be considered a valid
661 indicator of optimal gut health condition in sea bream.

662 No significant differences related to rearing density of any specific component within
663 each diet at phylum level were detected (Wilcoxon ran-sum test, $p > 0.05$, FDR

664 correction). However, different responses of the intestinal gut microbial composition in
665 relation to dietary treatment under high and low rearing density were detected as also
666 highlighted by weighted and unweighted UniFrac PCoA. In particular, no significant
667 separation was found between densities when fish were fed high FM-FO level, while
668 under low FM-FO diet density had a significant effect. Focusing on specific components
669 of the gut bacterial community, the results indicated that under high rearing density high
670 FM-FO level led to a significant increase in *Staphylococcaceae* and a reduction in
671 *Streptococcaceae* abundances compared to low FM-FO diet, while under low rearing
672 density *Lactobacillaceae* were less abundant in low FM-FO diet than high FM-FO diet.
673 Although no significant differences were detected, high rearing density seems to reduce
674 the amount of *Lactobacillaceae* (mainly *Lactobacillus spp*) within each dietary treatment
675 (Supplementary Table 1). No studies are available to compare the effect of rearing density
676 on specific gut microbial components in fish. In the present study, no evident signs of
677 stress induced by high rearing density were detected by results of performance, plasma
678 and skin mucus parameters; however, *Lactobacillaceae* may be highly sensitive in
679 relation to environmental stressors in fish and may deserve further attention for future
680 studies.

681 Analysis of biodiversity of the microbial community has highlighted a different
682 response to the feeding regimes, showing a general higher biodiversity in fish fed diets
683 containing higher vegetable ingredients. This is in general agreement with previous
684 findings detecting feeding habit as a key factor influencing fish gut microbial diversity
685 and observing an increasing trend in diversity following the order of carnivores,
686 omnivores and herbivores (Wang et al., 2018). In addition, a significant increase in α -
687 diversity indices at increasing FM replacement with vegetal ingredients was observed in

688 carnivorous fish species (Desai et al 2012; Miao et al., 2018). Concerning the interaction
689 between diet and rearing density, a low FM-FO diet maintained steady the biodiversity
690 of the ecosystem between low and high-density conditions while fish fed high FM-FO
691 level showed a significantly reduced biodiversity at high rearing density when compared
692 to the other groups. It has been suggested that in fish, reduction in diversity leads to
693 reduced competition for opportunistic or invading pathogens which may enter the
694 gastrointestinal tract of fish via feed or water (Apper et al., 2016). In several fish species,
695 α -diversity was not found to be affected by dietary vegetal ingredients (Apper et al., 2016;
696 Parma et al., 2016; Rimoldi et al., 2018b), by the interaction between diet and rearing
697 density (Wong et al., 2013) or by stocking density (Du et al., 2019). Also in pigs, stocking
698 density did not significantly affect biodiversity indices of gut microbiota (Li et al., 2017).
699 Interestingly, recent findings in the African cichlid *Astatotilapia burtoni* highlighted that
700 fish which experienced stressful conditions induced by subordinate social rank displayed
701 a reduced faecal microbial community α -diversity (Singh et al., 2019). Also in captive
702 mice and in wild red squirrels (*Tamiasciurus hudsonicus*) socially mediated stress
703 affected the intestinal microbiota leading to a reduction in microbial diversity and
704 richness (Bailey et al., 2011; Stothart et al., 2016). The reduction of biodiversity observed
705 in the present study only under the high FM-FO level could be correlated to increased
706 feeding competition only when a potentially more palatable high FM-FO diet is offered.
707 Another explanation may be associated with the lower feed intake observed under high
708 rearing density when fed high FM-FO level, or a combination of both factors: feeding
709 competition and feed intake. Recently, in perch (*Perca fluviatilis*) Zha et al. (2018) found
710 that gut microbial diversity responded to predation stress and food ration with a reduction
711 in diversity due to the presence of a predator and a reduced feed ration. The authors

712 suggested that a high ration of food favours bacteria that are quick colonizers and fast
713 growers while at lower food rations bacteria that are good competitors would be favoured.
714 In addition, the fact that in our study the reduction in gut microbial diversity was not
715 supported by evident altered physiological signs of stress could indicate a high sensitivity
716 of the gut microbial community structure to food competition, or to other social
717 interaction induced by rearing density. Thus, the analysis of gut microbial community
718 diversity could represent a valuable tool to assess social stress conditions for future
719 studies related to feeding behaviour and feeding competition.

720

721 **Conclusion**

722

723 In conclusion, the different rearing densities tested in this trial had no major effects on
724 overall performances and feed efficiency of gilthead sea bream reared at high or low fish
725 meal and fish oil dietary level. However, rearing density reduced feed intake in fish fed
726 high fish meal and fish oil dietary level. Results of digestive enzyme activities indicated
727 a comparable digestive efficiency among rearing densities and within dietary treatment
728 even if intestinal brush boarder enzymes such as LAP and aminopeptidase seems to be
729 more influenced by stocking density compared with other (gastric and pancreatic)
730 enzymes. Plasma parameters related to nutritional and physiological conditions were not
731 affected by rearing densities, indicating that sea bream can well cope with high rearing
732 density up to 36-44 kg m⁻³ and that a high level of vegetable dietary ingredients does not
733 amplify the potential stressful effects of rearing density. A similar observation was
734 achieved through the study of skin mucosal immunity; however in this case lysozyme
735 was slightly reduced at high density. For the first time the effect of rearing density on gut

736 bacterial community of this species was studied. Different responses in relation to dietary
737 treatment under high and low rearing density were detected. Low FM-FO diet maintained
738 steady the biodiversity of gut bacterial community between low and high rearing density
739 while fish fed high FM-FO level showed a significantly reduced biodiversity at high
740 rearing density possibly indicating higher social stress conditions related to feeding
741 competition under this treatment. According to the results, it seems feasible to rear
742 gilthead sea bream at the on-growing phase at a density up to 36-44 kg m⁻³ with low or
743 high FM-FO diet without negatively affecting growth, feed efficiency, welfare condition
744 and gut microbial community.

745

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747

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Table 1. Ingredients and proximate composition of the experimental diets

	FM30/FO15	FM10/FO3
<i>Ingredients, % of the diet</i>		
Fish meal (LT70)	30.0	10.0
Soybean meal 48	9.0	9.0
Soy protein concentrate	10.0	20.5
Wheat gluten	5.0	10.2
Corn gluten	10.0	15.0
Wheat meal	9.7	7.3
Rapeseed meal	5.0	4.0
Sunflower meal	5.0	4.0
Fish oil	15.0	3.0
Rapeseed oil	0	13.0
Vit/Min premix ¹	1.0	1.0
Antioxidant powder (Paramega)	0.2	0.2
Sodium propionate	0.1	0.1
MCP		2.0
Lysine	-	0.3
Methionine	-	0.1
L-Tryptophan		0.3
<i>Proximate composition, % on a wet weight basis</i>		
Moisture	5.83	4.9
Protein	46.3	44.7
Lipid	17.2	17.8
Ash	8.2	6.4
Gross energy cal g ⁻¹	4945.7	4823.6

¹Vitamins and mineral premix (IU or mg kg⁻¹ diet; Invivo NSA, Portugal); DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 16650 IU; DL-cholecalciferol, 2000 IU; thiamine, 25 mg; riboflavin, 25 mg; pyridoxine, 25 mg; cyanocobalamin, 0.1 mg; niacin, 150 mg; folic acid, 15 mg; L-ascorbic acid monophosphate, 750 mg; inositol, 500 mg; biotin, 0.75 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg; copper sulphate heptahydrate, 25 mg; ferric sulphate monohydrate, 100 mg; potassium iodide, 2 mg; manganese sulphate monohydrate, 100 mg; sodium selenite, 0.05 mg; zinc sulphate monohydrate, 200 mg
MCP: monocalcium phosphate

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Table 2. Growth performance of gilthead sea bream reared at low and high stocking density and fed the experimental diets over 98 days.

Experimental diets

	FM30/FO15		FM10/FO3		Density	<i>P value</i>	
	LD	HD	LD	HD		Diet	Inter
Initial density kg m ⁻³	4.8±0.1 ^a	14.5±0.6 ^b	4.8±0.1 ^a	14.3±0.1 ^b	<0.0001	0.7078	0.7078
Final density kg m ⁻³	15.2±0.5 ^b	43.6±0.5 ^d	12.1±1.3 ^a	35.9±0.5 ^c	<0.0001	<0.0001	0.0011
IBW(g)	96.1±1.1	96.4±3.7	96.6±2.6	95.5±0.8	0.768	0.878	0.630
FBW(g)	317.8±5.6 ^b	292.5±3.9 ^b	253.1±27.2 ^a	246.2±2.8 ^a	0.084	0.0001	0.292
Weight gain (g)	221.7±5.4 ^b	196.2±0.5 ^b	156.5±25.3 ^a	150.7±3.0 ^a	0.071	0.0001	0.224
SGR	1.22±0.02 ^b	1.13±0.03 ^b	0.98±0.09 ^a	0.97±0.02 ^a	0.127	0.0001	0.248
FI	15.6±0.19 ^b	14.6±0.21 ^a	15.4±0.64 ^{ab}	14.5±0.03 ^a	0.002	0.506	0.818
FCR	1.43±0.02 ^a	1.42±0.01 ^a	1.70±0.21 ^b	1.61±0.02 ^{ab}	0.433	0.005	0.495
Survival %	95.8±1.4 ^a	99.4±0.5 ^b	95.8±1.4 ^a	97.2±0.5 ^{ab}	0.004	0.111	0.111

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). FM30/FO15 = 300g kg⁻¹ fishmeal (FM), 150 g kg⁻¹ fish oil (FO); FM10/FO3 = 100g kg⁻¹ FM; 30g kg⁻¹ FO. LD, low rearing density; HD, high rearing density.

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).

FCR = feed conversion rate = feed intake (g) /weight gain (g)

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Table 3. Body composition and nutritional indices of gilthead sea bream reared at low and high stocking density and fed the experimental diets over 98 days.

	Experimental diets				<i>P</i> -value		
	FM30/FO15		FM10/FO3		<i>Density</i>	<i>Diet</i>	<i>Inter.</i>
	LD	HD	LD	HD			
Whole body composition, %							
Protein	17.0 ± 0.5	17.2 ± 0.1	17.0 ± 0.0	16.9 ± 0.1	0.835	0.333	0.358
Lipid	21.4 ± 2.5 ^b	19.5 ± 1.5 ^{ab}	16.6 ± 0.7 ^a	17.0 ± 0.8 ^a	0.451	0.003	0.233
Ash	3.43 ± 0.11	3.57 ± 0.25	3.88 ± 0.08	3.83 ± 0.21	0.662	0.008	0.37
Moisture	58.0 ± 0.49	58.7 ± 0.7	59.5 ± 0.8	60.3 ± 0.9	0.206	0.024	0.949
Nutritional indices							
PER	1.51 ± 0.02	1.52 ± 0.01	1.32 ± 0.16	1.39 ± 0.02	0.443	0.009	0.567
GPE	25.8 ± 0.88	26.4 ± 0.38	22.6 ± 2.74	23.4 ± 0.20	0.455	0.006	0.879
GLE	101 ± 14.8 ^b	91.7 ± 9.0 ^b	60.9 ± 9.4 ^a	66.2 ± 4.6 ^a	0.768	0.000	0.253
LER	4.08 ± 0.05 ^b	4.11 ± 0.03 ^b	3.32 ± 0.40 ^a	3.48 ± 0.04 ^a	0.476	0.000	0.579

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($p \leq 0.05$). FM30/FO15 = 300g kg⁻¹ fishmeal (FM), 150 g kg⁻¹ fish oil (FO); FM10/FO3 = 100g kg⁻¹ FM; 30g kg⁻¹ FO. LD, low rearing density; HD, high rearing density.

PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).

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

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

LER = Lipid efficiency ratio = ((FBW-IBW)/lipid intake).

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Table 4. Specific (U mg protein⁻¹) digestive enzyme activities of pancreatic (stomach and anterior intestine, AI) and intestinal brush border enzymes of gilthead sea bream reared at low (LD) and high (HD) stocking density and fed the experimental diets over 98 days.

	<i>Experimental diets</i>				<i>P-value</i>		
	FM30/FO15		FM10/FO3		<i>Density</i>	<i>Diet</i>	<i>Inter.</i>
	LD	HD	LD	HD			
<i>Pancreatic (Stomach/AI)</i>							
Pepsin	0.33 ± 0.11	0.34 ± 0.10	0.27 ± 0.18	0.55 ± 0.20	0.157	0.414	0.165
Trypsin	0.07 ± 0.03	0.04 ± 0.02	0.02 ± 0.02	0.03 ± 0.01	0.225	0.053	0.225
Chymotrypsin	0.60 ± 0.06	0.31 ± 0.17	0.34 ± 0.41	0.30 ± 0.20	0.276	0.366	0.413
Total alkaline proteases	0.56 ± 0.15	0.33 ± 0.15	0.25 ± 0.28	0.27 ± 0.13	0.333	0.119	0.270
Alpha-amylase	4.49 ± 1.47	3.38 ± 0.82	3.90 ± 3.24	2.37 ± 1.32	0.271	0.496	0.856
Bile salt activated lipase	0.017 ± 0.01	0.017 ± 0.01	0.022 ± 0.02	0.025 ± 0.01	0.784	0.264	0.819
<i>Brush border AI</i>							
Aminopeptidase-N	0.021 ± 0.01	0.022 ± 0.02	0.012 ± 0.01	0.008 ± 0.01	0.816	0.128	0.722
Phosphatase alkaline	1.83 ± 0.91	1.69 ± 0.31	1.10 ± 0.43	0.97 ± 0.09	0.701	0.075	0.981
Maltase	126.4 ± 25.8	124.1 ± 35.9	122.6 ± 36.9	64.9 ± 8.0	0.157	0.140	0.186
LAP	33.0 ± 3.1 ^{ab}	62.3 ± 18.7 ^b	24.7 ± 6.8 ^a	41.3 ± 4.8 ^{ab}	0.011	0.065	0.374
<i>Brush Border PI</i>							
Aminopeptidase	0.043 ± 0.01 ^b	0.026 ± 0.005 ^{ab}	0.0260 ± 0.005 ^{ab}	0.021 ± 0.005 ^a	0.031	0.031	0.169
Phosphatase alkaline	0.49 ± 0.10	0.94 ± 1.13	0.22 ± 0.08	0.13 ± 0.02	0.600	0.137	0.432
Maltase	130.5 ± 70.1	164.7 ± 62.9	64.8 ± 13.2	73.2 ± 26.1	0.524	0.042	0.700
LAP	46.6 ± 8.1 ^{ab}	45.9 ± 1.9 ^{ab}	55.6 ± 5.9 ^b	41.8 ± 0.9 ^a	0.038	0.430	0.058

Data are given as the mean (n = 3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($p \leq 0.05$). FM30/FO15 = 300g kg⁻¹ fishmeal (FM), 150 g kg⁻¹ fish oil (FO); FM10/FO3 = 100g kg⁻¹ FM; 30g kg⁻¹ FO. LD, low rearing density; HD, high rearing density, AI, anterior intestine; PI posterior intestine; LAP, leucine-alanine peptidase.

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Table 5. Plasma biochemistry values for sea bream kept under high (HD) and low (LD) rearing density and fed the experimental diets.

Parameters	<i>Experimental diets</i>						<i>P - value</i>	<i>Interaction</i>
	FM30/FO15		FM10/FO3		<i>Density</i>	<i>Diet</i>		
	LD	HD	LD	HD				
Glucose (mg dL ⁻¹)	119±26	123±29	117±31	101±24	0.374	0.079	0.145	
Urea (mg dL ⁻¹)	10.7±2.0 ^{ab}	9.25±1.44 ^a	11.6±2.1 ^{bc}	13.5±2.8 ^c	0.760	0.000	0.003	
Creatine (mg dL ⁻¹)	0.37±0.14 ^b	0.30±0.10 ^b	0.22±0.04 ^a	0.21±0.04 ^a	0.169	0.000	0.090	
Uric acid (mg dL ⁻¹)	0.51±0.40	0.39±0.25	0.42±0.42	0.32±0.30	0.206	0.361	0.868	
Tot bil (mg dL ⁻¹)	0.02±0.02	0.03±0.01	0.04±0.03	0.07±0.13	0.368	0.063	0.606	
Bil. Ac. (µmol dL ⁻¹)	69.3±39.7	64.8±41.7	48.9±30.4	61.2±40.8	0.685	0.215	0.381	
Amylase (U L ⁻¹)	2.88±5.35	0.88±0.34	1.25±1.00	1.50±2.12	0.226	0.488	0.121	
Lipase (U L ⁻¹)	2.20±2.43 ^a	1.69±1.74 ^a	4.13±2.92 ^{ab}	5.22±3.62 ^b	0.602	0.000	0.289	
CHOL (mg dL ⁻¹)	311±75 ^b	287±71 ^b	195±27 ^a	171±35 ^a	0.089	0.000	0.987	
TRIG (mg dL ⁻¹)	792±276	793±374	810±241	830±327	0.892	0.720	0.903	
TP (mg dL ⁻¹)	4.26±0.76 ^b	4.10±0.71 ^{ab}	3.78±0.29 ^{ab}	3.59±0.41 ^a	0.213	0.001	0.909	
ALB (g dL ⁻¹)	0.97±0.19 ^b	0.90±0.15 ^{ab}	0.89±0.06 ^{ab}	0.84±0.10 ^a	0.081	0.040	0.724	
AST(U L ⁻¹)	49.2±31.1	43.0±32.4	55.5±40.8	53.3±26.3	0.606	0.310	0.808	
ALT (U L ⁻¹)	1.81±1.76	1.31±0.60	1.19±0.54	1.11±0.32	0.232	0.088	0.378	
ALP (U L ⁻¹)	493±190	555±265	597±259	594±274	0.632	0.251	0.601	
CK (U L ⁻¹)	226±295	118±66	112±91	117±89	0.204	0.155	0.159	
GGT (U L ⁻¹)	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.0	1.000	1.000	1.000	
LDH (U L ⁻¹)	519±662	406±409	530±646	719±527	0.792	0.259	0.292	
Ca ⁺² (mg dL ⁻¹)	15.0±1.7 ^b	14.7±1.2 ^{ab}	14.3±0.7 ^{ab}	13.8±0.9 ^a	0.142	0.008	0.670	
P (mg dL ⁻¹)	13.3±2.1	12.0±1.8	12.2±1.4	12.3±2.4	0.249	0.381	0.183	
K ⁺ (mEq L ⁻¹)	7.16±2.45 ^b	5.28±1.58 ^a	7.06±1.70 ^{ab}	8.33±2.0 ^b	0.530	0.003	0.002	
Na ⁺ (mEq L ⁻¹)	188±6 ^a	189±5 ^{ab}	194±6 ^b	191±5 ^{ab}	0.566	0.005	0.094	
Fe (µg dL ⁻¹)	135±33	111±28	124±30	127±37	0.206	0.766	0.090	
Cl (mEq L ⁻¹)	148±4 ^a	150±4 ^a	157±5 ^b	156±4 ^b	0.325	0.000	0.131	
Mg (mg dL ⁻¹)	4.97±0.98 ^b	4.30±0.78 ^{ab}	3.86±0.50 ^a	3.86±0.72 ^a	0.078	0.000	0.073	
UIBC (µg dL ⁻¹)	464±78	433±97	502±68	488±96	0.300	0.031	0.695	
TIBC (µg dL ⁻¹)	599±97	544±116	626±74	616±105	0.193	0.049	0.373	
Cortisol (µg dL ⁻¹)	3.11±1.74	3.78±2.87	4.45±3.26	4.25±3.99	0.837	0.244	0.278	
ALB/GLOB	0.30±0.03 ^{ab}	0.28±0.02 ^a	0.31±0.02 ^b	0.31±0.02 ^b	0.174	0.002	0.158	
CaxP	201±50	178±39	175±24	169±36	0.138	0.068	0.366	
Na/K	28.9±8.8 ^a	38.8±10.7 ^b	29.1±7.5 ^a	24.1±6.0 ^a	0.243	0.001	0.001	

Data are given as the mean (n=15) ± SD. Different letters indicate significant difference ($P \leq 0.05$) between treatments. FM30/FO15 = 300g kg⁻¹ fishmeal (FM), 150 g kg⁻¹ fish oil (FO); FM10/FO3 = 100g kg⁻¹ FM; 30g kg⁻¹ FO. LD, low rearing density; HD, high rearing density. Tot Bil, total bilirubin; CHOL, cholesterol; TRIG, triglycerides; TP, total protein; ALB, albumin; AST, aspartate aminotransferase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; CK, creatine kinase; LDH, lactate dehydrogenase, Ca⁺², calcium; P, inorganic phosphorus; K⁺, potassium; Na⁺, sodium; Fe, iron; Cl, chloride; Mg, magnesium; UIBC, unsaturated iron binding capacity; TIBC, total iron binding capacity; GLOB, globuline.

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1067 **Key to Figures**

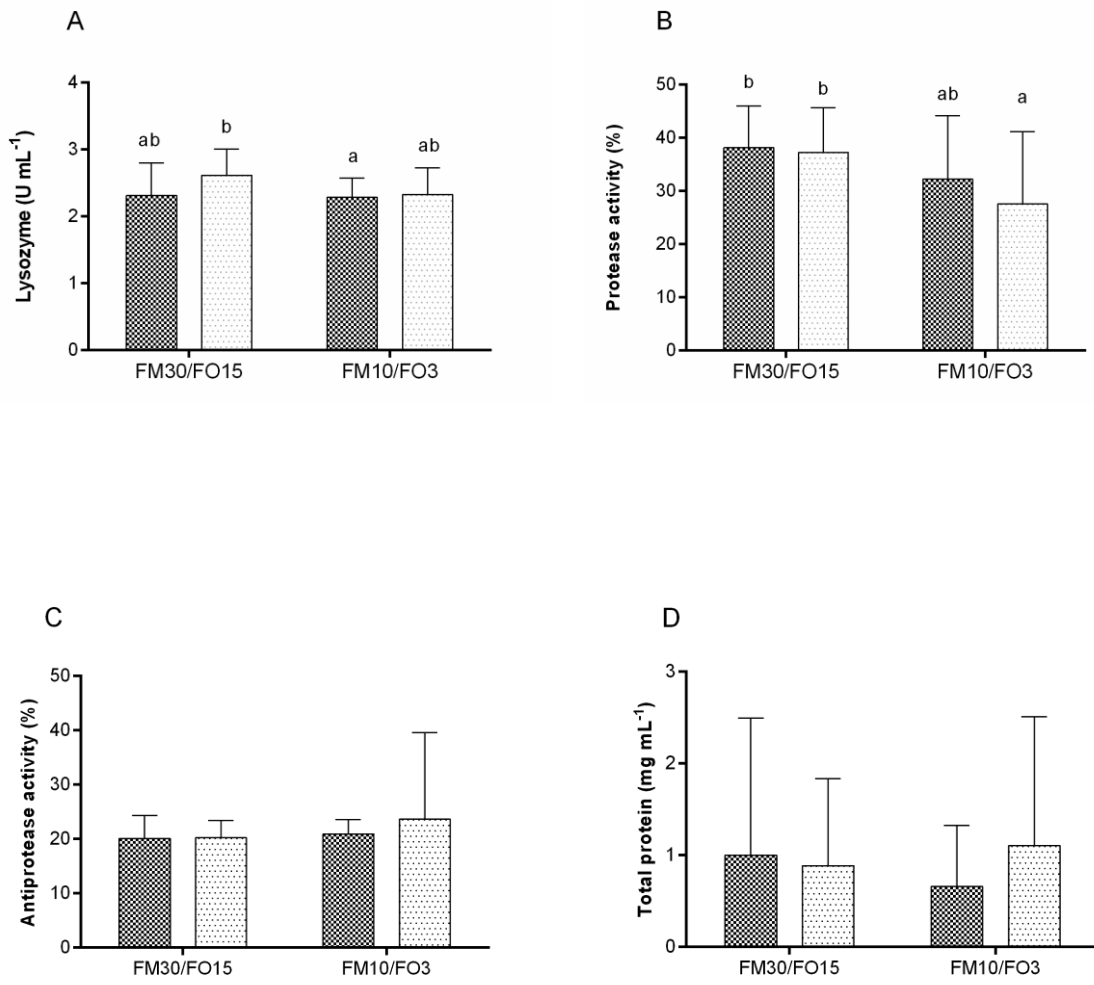
1068 Figure 1. A, Lysozyme (U mL^{-1}); B, protease activity (%); C, antiprotease activity (%);
1069 D, total protein (mg mL^{-1}) in skin mucus of gilthead seabream reared at low (LD, light
1070 grey) and high (HD, dark grey) stocking density and fed the experimental diets over 98
1071 days. FM30/FO15 = 300g kg^{-1} fishmeal (FM), 150 g kg^{-1} fish oil (FO); FM10/FO3 =
1072 100g kg^{-1} FM; 30g kg^{-1} FO. Data represent the mean \pm S.D. (N=24). Different letters
1073 denote significant differences between experimental groups ($p < 0.05$).

1074 Figure 2. Barplots representing the sea bream gut bacterial community at two
1075 phylogenetic levels: A) phylum; B) Family. In panel C) are reported the boxplots with
1076 the families showing a significant difference in relative abundance among groups (p value
1077 < 0.05 , Wilcoxon ran-sum test; FDR correction). FM30/FO15 = 300g kg^{-1} fishmeal (FM),
1078 150 g kg^{-1} fish oil (FO); FM10/FO3 = 100g kg^{-1} FM; 30g kg^{-1} FO. LD, low rearing
1079 density; HD, high rearing density.

1080 Figure 3. Internal biodiversity of sea bream gut microbiota in both feeding regimen and
1081 rearing densities computed using Hill numbers (A) highlighted a significant difference
1082 between diets ($p < 0.05$; Wilcoxon ran-sum test). Principal Coordinates Analysis
1083 (PCoA) plots obtained using weighted (B) and unweighted UniFrac (C) showing a
1084 significant difference among groups ($p < 0.01$; except FM30/FO15_{HD} vs FM30/FO15_{LD},
1085 $p > 0.05$; permutation test with pseudo-F ratios, Adonis). FM30/FO15 = 300g kg^{-1}
1086 fishmeal (FM), 150 g kg^{-1} fish oil (FO); FM10/FO3 = 100g kg^{-1} FM; 30g kg^{-1} FO. LD,
1087 low rearing density; HD, high rearing density.

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1089 Figure 1
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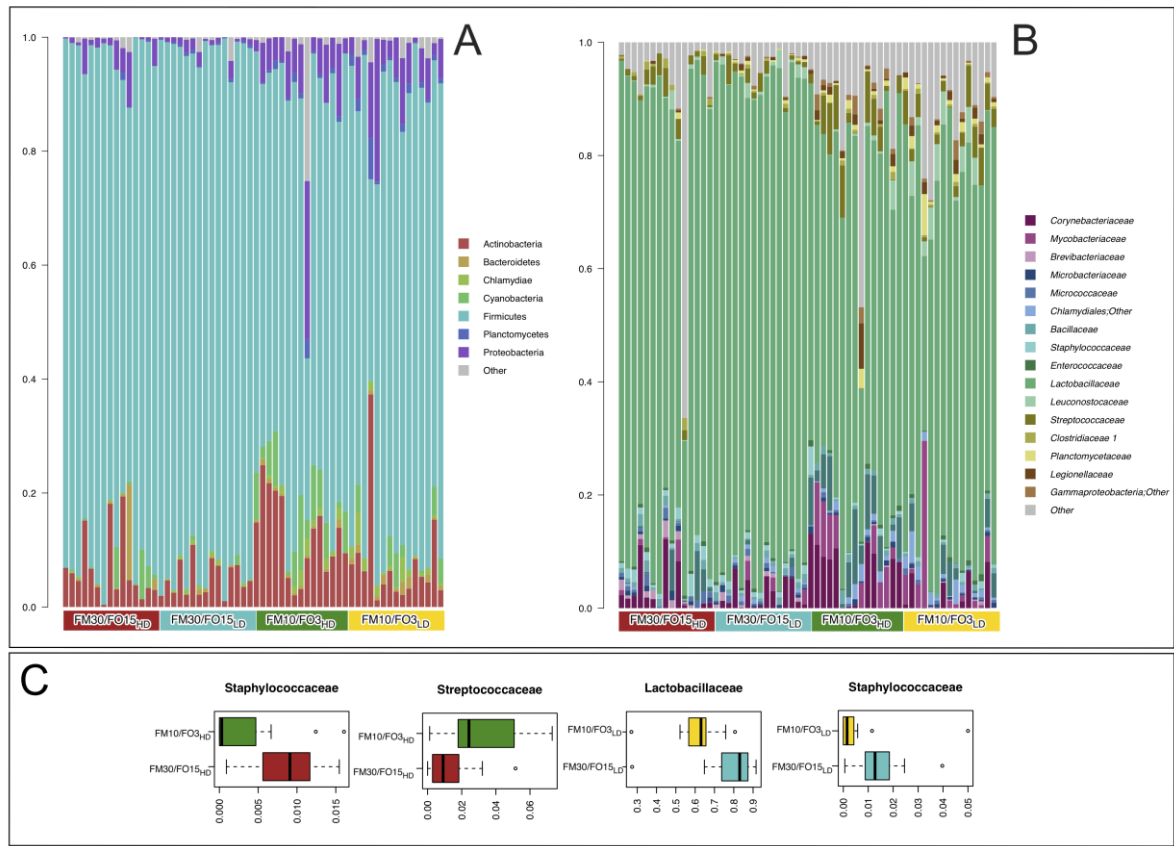
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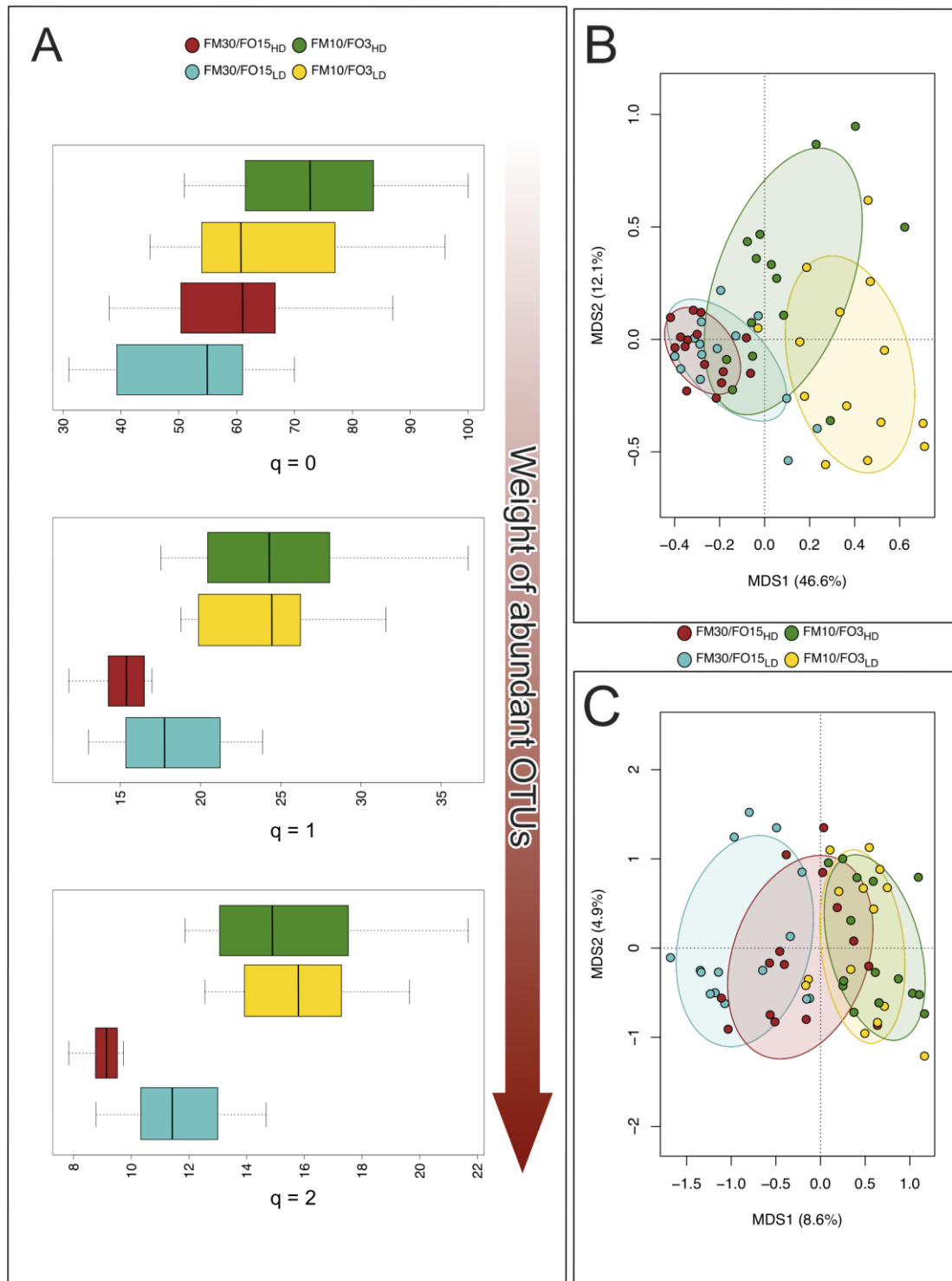
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1095 Figure 2



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Supplementary Table 1.

Mean relative abundance (%) \pm SD (n=15) of bacterial phyla, classes, orders, families and genera detected in the distal intestine content of gilthead sea bream fed different diets under high and low rearing density. FM30/FO15 = 300g kg⁻¹ fishmeal (FM), 150 g kg⁻¹ fish oil (FO); FM10/FO3 = 100g kg⁻¹ FM; 30g kg⁻¹ FO. LD, low rearing density; HD, high rearing density. Only taxa with mean relative abundance \geq 0.1% in at least 1 treatment were included.

Diet	FM30/FO15_{HD}		FM30/FO15_{LD}		FM10/FO3_{HD}		FM10/FO3_{LD}	
Phylum	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<i>Actinobacteria</i>	6.7	6.0	5.0	3.0	12.5	7.1	7.8	8.9
<i>Bacteroidetes</i>	1.4	4.3	0.2	0.3	0.5	0.4	0.9	0.7
<i>Chlamydiae</i>	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
<i>Chloroflexi</i>	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
<i>Cyanobacteria</i>	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5
<i>Firmicutes</i>	87.3	9.4	92.2	4.3	69.9	13.4	77.9	13.7
<i>Gracilibacteria</i>	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
<i>Lentisphaerae</i>	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0
<i>Planctomycetes</i>	0.1	0.3	0.3	0.2	0.8	0.9	1.4	1.7
<i>Proteobacteria</i>	2.5	2.9	1.2	0.9	7.6	6.3	7.1	6.1
<i>Saccharibacteria</i>	0.3	0.5	0.2	0.4	0.6	1.3	0.8	1.1
<i>Spirochaetae</i>	0.1	0.4	0.0	0.0	0.5	1.7	0.0	0.0
<i>TM6 (Dependentiae)</i>	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.2
<i>Verrucomicrobia</i>	0.0	0.1	0.0	0.2	0.1	0.3	0.0	0.0
<i>WS6</i>	0.0	0.0	0.0	0.1	0.2	0.7	0.0	0.1
<i>Unassigned;Other</i>	0.0	0.1	0.1	0.2	0.2	0.8	0.2	0.4
Class								
<i>Acidimicrobiia</i>	0.0	0.0	0.1	0.1	0.5	0.9	0.9	1.6
<i>Actinobacteria</i>	6.0	5.6	4.9	3.0	11.6	7.1	6.7	7.7
<i>Coriobacteriia</i>	0.4	0.9	0.0	0.1	0.4	0.8	0.1	0.3
<i>Thermoleophilia</i>	0.3	1.0	0.0	0.0	0.1	0.2	0.0	0.0
<i>Bacteroidia</i>	1.3	4.3	0.1	0.2	0.1	0.1	0.4	0.5
<i>Flavobacteriia</i>	0.1	0.2	0.1	0.1	0.3	0.2	0.4	0.5
<i>Sphingobacteriia</i>	0.0	0.0	0.0	0.0	0.2	0.3	0.2	0.2
<i>Chlamydiae</i>	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
<i>Chloroflexi;KD4-96</i>	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
<i>Chloroplast</i>	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5
<i>Bacilli</i>	83.6	16.2	91.1	4.4	68.2	13.1	75.5	13.4
<i>Clostridia</i>	3.2	7.0	1.0	0.5	1.5	1.6	2.1	1.2
<i>Erysipelotrichia</i>	0.2	0.6	0.0	0.0	0.0	0.0	0.1	0.1
<i>Negativicutes</i>	0.3	0.8	0.0	0.1	0.2	0.3	0.3	0.4
<i>Gracilibacteria;Other</i>	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
<i>Planctomycetacia</i>	0.1	0.3	0.3	0.2	0.8	0.9	1.4	1.7
<i>Alphaproteobacteria</i>	0.6	1.1	0.3	0.3	2.5	2.3	2.0	2.6

<i>Betaproteobacteria</i>	0.2	0.4	0.1	0.1	0.5	0.7	0.1	0.2
<i>Deltaproteobacteria</i>	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>Epsilonproteobacteria</i>	0.1	0.2	0.0	0.0	0.0	0.0	0.2	0.6
<i>Gammaproteobacteria</i>	1.5	2.4	0.8	0.6	4.5	4.5	4.8	5.8
<i>Saccharibacteria;uncultured bacterium</i>	0.3	0.5	0.2	0.4	0.6	1.3	0.8	1.1
<i>Spirochaetes</i>	0.1	0.4	0.0	0.0	0.5	1.7	0.0	0.0
<i>TM6 (Dependentiae);uncultured bacterium</i>	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.2
<i>Verrucomicrobiae</i>	0.0	0.0	0.0	0.2	0.1	0.3	0.0	0.0
Order								
<i>Acidimicrobiales</i>	0.0	0.0	0.1	0.1	0.5	0.9	0.9	1.6
<i>Bifidobacteriales</i>	0.2	0.5	0.1	0.1	0.3	0.7	0.3	0.4
<i>Corynebacteriales</i>	3.4	4.8	3.0	3.0	10.0	7.1	5.2	7.8
<i>Micrococcales</i>	2.3	1.4	1.5	0.6	0.8	0.6	0.9	0.4
<i>Propionibacteriales</i>	0.0	0.1	0.2	0.2	0.4	1.1	0.3	0.3
<i>Streptomycetales</i>	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
<i>Coriobacteriales</i>	0.4	0.9	0.0	0.1	0.4	0.8	0.1	0.3
<i>Solirubrobacterales</i>	0.2	0.8	0.0	0.0	0.1	0.2	0.0	0.0
<i>Bacteroidales</i>	1.3	4.3	0.1	0.2	0.1	0.1	0.4	0.5
<i>Flavobacteriales</i>	0.1	0.2	0.1	0.1	0.3	0.2	0.4	0.5
<i>Sphingobacteriales</i>	0.0	0.0	0.0	0.0	0.2	0.3	0.2	0.2
<i>Chlamydiales</i>	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
<i>Chloroflexi;KD4-96;uncultured bacterium</i>	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
<i>Chloroplast;Other</i>	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5
<i>Bacillales</i>	2.8	1.1	1.8	0.7	1.7	1.2	1.1	0.7
<i>Lactobacillales</i>	80.8	15.9	89.2	4.2	66.4	12.7	74.4	13.1
<i>Clostridiales</i>	3.2	7.0	1.0	0.5	1.5	1.6	2.1	1.2
<i>Erysipelotrichales</i>	0.2	0.6	0.0	0.0	0.0	0.0	0.1	0.1
<i>Selenomonadales</i>	0.3	0.8	0.0	0.1	0.2	0.3	0.3	0.4
<i>Gracilibacteria;Other</i>	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
<i>Planctomycetales</i>	0.1	0.3	0.3	0.2	0.8	0.9	1.4	1.7
<i>Rhizobiales</i>	0.2	0.8	0.2	0.2	1.5	2.0	1.2	2.5
<i>Rhodobacterales</i>	0.0	0.1	0.0	0.1	0.2	0.3	0.3	0.4
<i>Rhodospirillales</i>	0.0	0.0	0.0	0.0	0.1	0.1	0.3	0.9
<i>Rickettsiales</i>	0.3	0.6	0.1	0.2	0.6	0.8	0.2	0.4
<i>Sphingomonadales</i>	0.0	0.2	0.0	0.0	0.1	0.2	0.1	0.1
<i>Burkholderiales</i>	0.2	0.4	0.1	0.1	0.5	0.7	0.1	0.2
<i>Campylobacterales</i>	0.1	0.2	0.0	0.0	0.0	0.0	0.2	0.6
<i>Aeromonadales</i>	0.5	1.6	0.0	0.0	0.0	0.0	0.0	0.0
<i>Enterobacteriales</i>	0.3	0.7	0.1	0.1	0.2	0.3	0.2	0.3
<i>Gammaproteobacteria;HTA4</i>	0.1	0.2	0.0	0.1	0.4	1.0	0.3	0.6

<i>Legionellales</i>	0.2	0.2	0.3	0.4	1.9	2.7	1.4	1.1
<i>Pseudomonadales</i>	0.1	0.3	0.1	0.1	0.2	0.3	0.2	0.2
<i>Vibrionales</i>	0.3	1.2	0.0	0.1	0.3	0.6	1.6	6.1
<i>Xanthomonadales</i>	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.3
<i>Gammaproteobacteria; Other</i>	0.0	0.1	0.1	0.2	1.2	1.1	0.8	1.0
<i>Saccharibacteria; uncultured bacterium</i>	0.3	0.5	0.2	0.4	0.6	1.3	0.8	1.1
<i>Spirochaetales</i>	0.1	0.4	0.0	0.0	0.5	1.7	0.0	0.0
<i>TM6 (Dependentiae); uncultured bacterium;</i>	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.2
<i>Verrucomicrobiales</i>	0.0	0.0	0.0	0.2	0.1	0.3	0.0	0.0
<i>WS6; Other</i>	0.0	0.0	0.0	0.1	0.1	0.6	0.0	0.0
<i>Unassigned; Other</i>	0.0	0.1	0.1	0.2	0.2	0.8	0.2	0.4
Family								
<i>Acidimicrobiales; OM1 clade</i>	0.0	0.0	0.0	0.0	0.2	0.4	0.4	0.8
<i>Acidimicrobiales; uncultured</i>	0.0	0.0	0.0	0.0	0.1	0.2	0.4	1.3
<i>Bifidobacteriaceae</i>	0.2	0.5	0.1	0.1	0.3	0.7	0.3	0.4
<i>Corynebacteriaceae</i>	2.9	4.1	2.4	2.6	6.1	5.2	1.8	2.9
<i>Mycobacteriaceae</i>	0.4	1.0	0.6	1.2	3.9	3.9	3.3	7.5
<i>Brevibacteriaceae</i>	0.8	0.9	0.4	0.5	0.0	0.1	0.1	0.2
<i>Dermabacteraceae</i>	0.1	0.2	0.1	0.2	0.0	0.0	0.0	0.1
<i>Intrasporangiaceae</i>	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1
<i>Microbacteriaceae</i>	0.5	0.5	0.3	0.3	0.5	0.4	0.4	0.3
<i>Micrococcaceae</i>	0.9	0.8	0.6	0.4	0.2	0.3	0.3	0.3
<i>Nocardoidaceae</i>	0.0	0.0	0.0	0.0	0.3	1.0	0.0	0.0
<i>Propionibacteriaceae</i>	0.0	0.1	0.2	0.2	0.1	0.2	0.2	0.3
<i>Coriobacteriaceae</i>	0.4	0.9	0.0	0.1	0.4	0.8	0.1	0.3
<i>Solirubrobacterales; Elev-16S-1332</i>	0.2	0.8	0.0	0.0	0.0	0.0	0.0	0.0
<i>Bacteroidaceae</i>	0.1	0.4	0.0	0.1	0.0	0.1	0.2	0.4
<i>Prevotellaceae</i>	0.9	3.1	0.0	0.1	0.0	0.0	0.1	0.2
<i>Flavobacteriaceae</i>	0.1	0.1	0.1	0.1	0.3	0.2	0.4	0.4
<i>Chitinophagaceae</i>	0.0	0.0	0.0	0.0	0.2	0.3	0.1	0.2
<i>Chlamydiales; Other</i>	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
<i>Chloroflexi; KD4-96; uncultured bacterium</i>	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
<i>Chloroplast; Other</i>	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5
<i>Bacillaceae</i>	1.0	0.7	0.8	0.5	0.9	0.6	0.6	0.4
<i>Paenibacillaceae</i>	0.1	0.2	0.0	0.1	0.1	0.2	0.1	0.1
<i>Planococcaceae</i>	0.1	0.2	0.1	0.1	0.2	0.2	0.1	0.1
<i>Staphylococcaceae</i>	1.4	1.0	0.9	0.4	0.6	1.3	0.3	0.5
<i>Bacillales; Other</i>	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>Aerococcaceae</i>	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.1
<i>Carnobacteriaceae</i>	0.0	0.1	0.0	0.1	0.1	0.2	0.1	0.2

<i>Enterococcaceae</i>	0.4	0.3	0.3	0.2	0.4	0.4	0.4	0.4
<i>Lactobacillaceae</i>	77.9	16.1	86.5	4.4	61.3	12.4	67.6	12.2
<i>Leuconostocaceae</i>	0.5	0.8	1.0	1.1	0.5	1.3	3.0	2.7
<i>Streptococcaceae</i>	2.0	1.5	1.3	1.4	4.1	3.7	3.2	2.3
<i>Clostridiaceae 1</i>	0.7	0.7	0.2	0.2	0.4	0.4	0.4	0.2
<i>Clostridiaceae 2</i>	0.0	0.0	0.0	0.0	0.1	0.5	0.0	0.0
<i>Clostridiales; Family XI</i>	0.3	0.3	0.3	0.2	0.2	0.2	0.3	0.3
<i>Clostridiales; Family XIII</i>	0.1	0.2	0.1	0.3	0.0	0.0	0.0	0.0
<i>Lachnospiraceae</i>	0.6	2.1	0.2	0.3	0.1	0.2	0.4	0.5
<i>Peptostreptococcaceae</i>	0.2	0.3	0.1	0.1	0.1	0.2	0.1	0.2
<i>Ruminococcaceae</i>	1.1	4.1	0.0	0.1	0.0	0.0	0.4	0.6
<i>Clostridiales; Other</i>	0.1	0.3	0.1	0.2	0.6	0.9	0.5	0.8
<i>Erysipelotrichaceae</i>	0.2	0.6	0.0	0.0	0.0	0.0	0.1	0.1
<i>Acidaminococcaceae</i>	0.2	0.6	0.0	0.0	0.0	0.0	0.1	0.3
<i>Veillonellaceae</i>	0.2	0.3	0.0	0.1	0.2	0.3	0.1	0.2
<i>Gracilibacteria; Other</i>	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
<i>Planctomycetaceae</i>	0.1	0.3	0.3	0.2	0.8	0.9	1.4	1.7
<i>Bradyrhizobiaceae</i>	0.0	0.0	0.0	0.0	0.7	1.6	0.1	0.3
<i>Brucellaceae</i>	0.0	0.0	0.0	0.0	0.2	0.5	0.1	0.2
<i>Hyphomicrobiaceae</i>	0.1	0.2	0.0	0.0	0.0	0.1	0.1	0.5
<i>Phyllobacteriaceae</i>	0.2	0.5	0.1	0.2	0.2	0.5	0.5	1.8
<i>Rhizobiaceae</i>	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.3
<i>Rhizobiales; Other</i>	0.0	0.0	0.0	0.1	0.2	0.3	0.2	0.2
<i>Rhodobacteraceae</i>	0.0	0.1	0.0	0.1	0.2	0.3	0.3	0.4
<i>Acetobacteraceae</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.9
<i>Mitochondria</i>	0.3	0.6	0.1	0.2	0.6	0.8	0.2	0.4
<i>Sphingomonadaceae</i>	0.0	0.2	0.0	0.0	0.1	0.2	0.1	0.1
<i>Comamonadaceae</i>	0.2	0.4	0.0	0.1	0.3	0.4	0.1	0.2
<i>Oxalobacteraceae</i>	0.0	0.0	0.0	0.1	0.2	0.4	0.0	0.1
<i>Helicobacteraceae</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.6
<i>Aeromonadaceae</i>	0.2	0.5	0.0	0.0	0.0	0.0	0.0	0.0
<i>Succinivibrionaceae</i>	0.3	1.3	0.0	0.0	0.0	0.0	0.0	0.0
<i>Enterobacteriaceae</i>	0.3	0.7	0.1	0.1	0.2	0.3	0.2	0.3
<i>Gammaproteobacteria; HTA4; Other</i>	0.1	0.2	0.0	0.1	0.4	1.0	0.3	0.6
<i>Coxiellaceae</i>	0.1	0.2	0.1	0.1	0.5	0.9	0.4	0.4
<i>Legionellaceae</i>	0.1	0.2	0.3	0.3	1.4	2.0	1.0	0.9
<i>Moraxellaceae</i>	0.1	0.3	0.1	0.1	0.2	0.2	0.1	0.3
<i>Vibrionaceae</i>	0.3	1.2	0.0	0.1	0.3	0.6	1.6	6.1
<i>Xanthomonadaceae</i>	0.0	0.0	0.0	0.0	0.1	0.2	0.2	0.3
<i>Gammaproteobacteria; Other</i>	0.0	0.1	0.1	0.2	1.2	1.1	0.8	1.0
<i>Saccharibacteria; uncultured bacterium</i>	0.3	0.5	0.2	0.4	0.6	1.3	0.8	1.1
<i>Brevinemataceae</i>	0.0	0.0	0.0	0.0	0.5	1.7	0.0	0.0

<i>TM6 (Dependentiae); uncultured bacterium</i>	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.2
<i>Verrucomicrobiaceae</i>	0.0	0.0	0.0	0.2	0.1	0.2	0.0	0.0
<i>WS6;Other</i>	0.0	0.0	0.0	0.1	0.1	0.6	0.0	0.0
<i>Unassigned;Other</i>	0.0	0.1	0.1	0.2	0.2	0.8	0.2	0.4
Genus								
<i>Acidimicrobiales; OMI clade; uncultured bacterium</i>	0.0	0.0	0.0	0.0	0.2	0.4	0.4	0.8
<i>Acidimicrobiales; uncultured;Other</i>	0.0	0.0	0.0	0.0	0.0	0.1	0.4	1.3
<i>Bifidobacterium</i>	0.2	0.5	0.1	0.1	0.3	0.7	0.3	0.4
<i>Corynebacterium 1</i>	2.8	4.1	2.3	2.5	6.1	5.2	1.8	2.9
<i>Mycobacterium</i>	0.4	1.0	0.6	1.2	3.9	3.9	3.3	7.5
<i>Nocardia</i>	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
<i>Brevibacterium</i>	0.8	0.9	0.4	0.5	0.0	0.1	0.1	0.2
<i>Brachybacterium</i>	0.1	0.2	0.1	0.2	0.0	0.0	0.0	0.1
<i>Intrasporangiaceae;Other</i>	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1
<i>Leucobacter</i>	0.3	0.4	0.1	0.2	0.2	0.4	0.1	0.2
<i>Microbacteriaceae;Other</i>	0.2	0.5	0.2	0.3	0.3	0.4	0.2	0.3
<i>Arthrobacter</i>	0.2	0.4	0.3	0.4	0.1	0.2	0.1	0.2
<i>Glutamicibacter</i>	0.2	0.6	0.1	0.2	0.0	0.1	0.0	0.1
<i>Kocuria</i>	0.3	0.4	0.2	0.2	0.1	0.3	0.2	0.2
<i>Micrococcaceae;Other</i>	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.1
<i>Nocardioides</i>	0.0	0.0	0.0	0.0	0.3	1.0	0.0	0.0
<i>Propionibacterium</i>	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.3
<i>Collinsella</i>	0.2	0.8	0.0	0.0	0.0	0.0	0.0	0.1
<i>Enterorhabdus</i>	0.0	0.1	0.0	0.0	0.3	0.8	0.0	0.0
<i>Coriobacteriaceae; uncultured</i>	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0
<i>Solirubrobacterales; Elev-16S-1332 uncultured bacterium</i>	0.2	0.8	0.0	0.0	0.0	0.0	0.0	0.0
<i>Bacteroides</i>	0.1	0.4	0.0	0.1	0.0	0.1	0.2	0.4
<i>Bacteroidales S24-7 group; uncultured bacterium</i>	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
<i>Prevotella 2</i>	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0
<i>Prevotella 9</i>	0.7	2.2	0.0	0.1	0.0	0.0	0.1	0.2
<i>Cloacibacterium</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
<i>Flavobacterium</i>	0.0	0.1	0.0	0.0	0.1	0.2	0.2	0.2
<i>Flavobacteriaceae;Other</i>	0.0	0.1	0.0	0.1	0.1	0.2	0.1	0.3
<i>Sediminibacterium</i>	0.0	0.0	0.0	0.0	0.1	0.2	0.1	0.2
<i>Chlamydiales;Other</i>	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
<i>Chloroflexi; KD4-96; uncultured bacterium</i>	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
<i>Chloroplast;Other</i>	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5

<i>Bacillus</i>	0.5	0.4	0.5	0.4	0.8	0.6	0.5	0.4
<i>Bacillaceae;Other</i>	0.4	0.3	0.3	0.3	0.0	0.1	0.1	0.1
<i>Brevibacillus</i>	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1
<i>Paenibacillus</i>	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.1
<i>Planococcaceae;Other</i>	0.1	0.1	0.1	0.1	0.2	0.2	0.0	0.1
<i>Staphylococcus</i>	1.3	1.0	0.8	0.4	0.5	1.3	0.3	0.5
<i>Staphylococcaceae;Other</i>	0.1	0.2	0.1	0.2	0.1	0.2	0.0	0.1
<i>Bacillales;Other</i>	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>Granulicatella</i>	0.0	0.1	0.0	0.1	0.1	0.2	0.1	0.2
<i>Enterococcus</i>	0.3	0.3	0.3	0.2	0.4	0.4	0.4	0.4
<i>Lactobacillus</i>	77.9	16.1	86.5	4.4	61.3	12.4	67.6	12.2
<i>Leuconostoc</i>	0.0	0.1	0.3	0.3	0.1	0.2	0.1	0.3
<i>Weissella</i>	0.4	0.8	0.7	0.9	0.4	1.3	2.8	2.8
<i>Lactococcus</i>	0.3	0.4	0.1	0.2	0.5	0.4	0.3	0.3
<i>Streptococcus</i>	1.6	1.6	1.2	1.4	3.6	3.5	2.9	2.2
<i>Clostridium sensu stricto 1</i>	0.4	0.7	0.1	0.1	0.0	0.1	0.1	0.1
<i>Clostridiaceae 1;Other</i>	0.2	0.4	0.1	0.2	0.3	0.4	0.2	0.3
<i>Alkaliphilus</i>	0.0	0.0	0.0	0.0	0.1	0.5	0.0	0.0
<i>Clostridiales; Family XI;uncultured</i>	0.1	0.2	0.0	0.1	0.0	0.2	0.0	0.1
<i>Clostridiales; Family XI;Other</i>	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.3
<i>Blautia</i>	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.2
<i>Roseburia</i>	0.2	0.6	0.0	0.2	0.0	0.0	0.0	0.0
<i>Peptostreptococcaceae; Other</i>	0.2	0.3	0.0	0.1	0.1	0.2	0.0	0.1
<i>Faecalibacterium</i>	0.3	1.0	0.0	0.1	0.0	0.0	0.1	0.3
<i>Ruminococcaceae UCG-002</i>	0.1	0.6	0.0	0.0	0.0	0.0	0.0	0.1
<i>Ruminococcaceae UCG-005</i>	0.2	0.7	0.0	0.0	0.0	0.0	0.0	0.0
<i>Ruminococcus 2</i>	0.1	0.4	0.0	0.1	0.0	0.0	0.0	0.1
<i>[Eubacterium] coprostanoligenes group</i>	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>Ruminococcaceae; uncultured</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.3
<i>Clostridiales; Other</i>	0.1	0.3	0.1	0.2	0.6	0.9	0.5	0.8
<i>Phascolarctobacterium</i>	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.1
<i>Acidaminococcaceae;Other</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2
<i>Megasphaera</i>	0.0	0.1	0.0	0.1	0.2	0.2	0.1	0.1
<i>Gracilibacteria; Othe</i>	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
<i>Planctomycetaceae; Pir4 lineage</i>	0.0	0.0	0.1	0.1	0.2	0.2	0.2	0.0
<i>Planctomyces</i>	0.0	0.1	0.1	0.2	0.3	0.3	0.6	0.0
<i>Planctomycetaceae; uncultured</i>	0.1	0.3	0.1	0.2	0.2	0.4	0.4	0.1
<i>Bradyrhizobium</i>	0.0	0.0	0.0	0.0	0.7	1.6	0.1	0.0
<i>Ochrobactrum</i>	0.0	0.0	0.0	0.0	0.2	0.5	0.1	0.0
<i>Hyphomicrobium</i>	0.1	0.2	0.0	0.0	0.0	0.0	0.1	0.0
<i>Mesorhizobium</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
<i>Phyllobacteriaceae; Other</i>	0.2	0.5	0.1	0.2	0.2	0.5	0.4	0.4

<i>Rhizobiales; Other</i>	0.0	0.0	0.0	0.1	0.2	0.3	0.2	0.2
<i>Rhodobacteraceae; Other</i>	0.0	0.0	0.0	0.1	0.1	0.2	0.2	0.0
<i>Acetobacteraceae; Other</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1
<i>Mitochondria;Other</i>	0.3	0.6	0.1	0.2	0.6	0.8	0.2	0.0
<i>Delftia</i>	0.0	0.0	0.0	0.1	0.3	0.4	0.1	0.0
<i>Comamonadaceae;Other</i>	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>Oxalobacteraceae;Other</i>	0.0	0.0	0.0	0.1	0.2	0.4	0.0	0.0
<i>Succinivibrio</i>	0.3	1.3	0.0	0.0	0.0	0.0	0.0	0.6
<i>Escherichia-Shigella</i>	0.2	0.7	0.0	0.0	0.0	0.1	0.1	0.0
<i>Serratia</i>	0.1	0.2	0.0	0.0	0.2	0.2	0.1	0.0
<i>Gammaproteobacteria; HTA4;Other</i>	0.1	0.2	0.0	0.1	0.4	1.0	0.3	0.0
<i>Aquicella</i>	0.0	0.1	0.0	0.1	0.2	0.4	0.2	0.2
<i>Coxiella</i>	0.1	0.2	0.0	0.1	0.3	0.5	0.2	0.0
<i>Legionella</i>	0.1	0.2	0.3	0.3	1.3	2.0	0.8	0.3
<i>Legionellaceae; Other</i>	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0
<i>Acinetobacter</i>	0.1	0.2	0.1	0.1	0.2	0.2	0.1	0.5
<i>Photobacterium</i>	0.2	1.0	0.0	0.0	0.2	0.6	0.4	0.0
<i>Vibrio</i>	0.1	0.2	0.0	0.1	0.0	0.0	1.2	0.2
<i>Stenotrophomonas</i>	0.0	0.0	0.0	0.0	0.1	0.2	0.1	0.1
<i>Gammaproteobacteria;Other;</i>	0.0	0.1	0.1	0.2	1.2	1.1	0.8	4.6
<i>Saccharibacteria; uncultured bacterium;</i>	0.3	0.5	0.2	0.4	0.6	1.3	0.8	0.1
<i>Brevinema</i>	0.0	0.0	0.0	0.0	0.5	1.7	0.0	0.1
<i>TM6 (Dependentiae); uncultured bacterium</i>	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.1
<i>WS6;Other</i>	0.0	0.0	0.0	0.1	0.1	0.6	0.0	0.0
<i>Unassigned;Other</i>	0.0	0.1	0.1	0.2	0.2	0.8	0.2	0.0