

Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Effects of rearing density on growth, digestive conditions, welfare indicators and gut bacterial community of gilthead sea bream (Sparus aurata, L. 1758) fed different fishmeal and fish oil dietary levels / Luca Parma, Nicole Francesca Pelusio, Enric Gisbert, Maria Angeles Esteban, Federica D'Amico, Matteo Soverini, Marco Candela, Francesco Dondi, Pier Paolo Gatta, Alessio Bonaldo. - In: AQUACULTURE. - ISSN 0044-8486. - ELETTRONICO. - 518:a-head of print:15 March 2020(2020), pp. 734854.1-734854.13.

This version is available at: https://hdl.handle.net/11585/716908 since: 2020-02-25

Published:

DOI: http://doi.org/10.1016/j.aquaculture.2019.734854

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/). When citing, please refer to the published version.

1	
2	
3	This is the final peer-reviewed accepted manuscript of:
4	Parma, L., Pelusio N.F., Gisbert, E., Esteban, M.A., D'Amico, F., Soverini,
5	M., Candela, M., Dondi, F., Gatta, P.P., Bonaldo, A. 2020. Effects of rearing
6	density on growth, digestive conditions, welfare indicators and gut bacterial
7	community of gilthead sea bream (Sparus aurata, L. 1758) fed different
8	fishmeal and fish oil dietary levels. Aquaculture, 518, 734850
9	The final published version is available online at:
9 10	The final published version is available online at: https://doi.org/10.1016/j.aquaculture.2019.734854
9 10 11	The final published version is available online at: https://doi.org/10.1016/j.aquaculture.2019.734854
9 10 11 12 13 14	The final published version is available online at: https://doi.org/10.1016/j.aquaculture.2019.734854 © [2020]. This manuscript version is made available under the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) 4.0 International License (http://creativecommons.org/licenses/by-nc-nd/4.0/)
9 10 11 12 13 14 15	The final published version is available online at: https://doi.org/10.1016/j.aquaculture.2019.734854 © [2020]. This manuscript version is made available under the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) 4.0 International License (http://creativecommons.org/licenses/by-nc-nd/4.0/)

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	
22	Luca Parma ^a *, Nicole Francesca Pelusio ^a , Enric Gisbert ^b , Maria Angeles Esteban ^c ,
23	Federica D'Amico ^d , Matteo Soverini ^d , Marco Candela ^d , Francesco Dondi ^a , Pier Paolo
24	Gatta ^a , Alessio Bonaldo ^a
25	
26	^a Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra
27	50, 40064 Ozzano Emilia, Italy
28	^b IRTA – Sant Carles de la Ràpita, Programa d'Aqüicultura, Crta. del Poble Nou km 5.5,
29	43540 Sant Carles de la Ràpita, Spain
30	^c Department of Cell Biology and Histology, Faculty of Biology, Campus Regional de
31	Excelencia Internacional "Campus Mare Nostrum", University of Murcia, 30100 Murcia,
32	Spain
33	^d Unit of Microbial Ecology of Health, Department of Pharmacy and Biotechnology,
34	University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy
35	
36	*Corresponding author: Luca Parma, Department of Veterinary Medical Sciences,
37	University of Bologna, Viale Vespucci 2, 47042 Cesenatico, FC, Italy. Tel.: +39 0547
38	338931; <i>Fax</i> : +39 0547 338941
39	E-mail address: <u>luca.parma@unibo.it</u> (L. Parma)
40	

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 kg m⁻³) and low (LD, 12-15 kg m⁻³) final stocking densities and fed high (FM30/FO15, 52 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 lysozyme was slightly reduced at HD. For the first time on this species, the effect of 65

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

73

74 Keywords

75

Gilthead sea bream, rearing density, fishmeal and fish oil replacement, digestive
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 recently high stocking density (final density 57 kg m^{-3}) decreased growth performance, 99 100 feed intake and feed efficiency of gilthead sea bream (12-58 g) in comparison to lower density 5-26 kg m⁻³ (Diogenes et al., 2019). In addition, in adult fish (272-425g) rearing 101 102 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 et al., 2017; Guardiola et al., 2018; Skrzynska et al., 2018; Diógenes et al., 2019). Most 109 110 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

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

136

137 2.2 Fish density and rearing

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna (Cesenatico, Italy). Gilthead sea bream were obtained from the fish farm Cosa s.r.l (Orbello, GR) and adapted to the laboratory facilities for 10 days before the beginning of the trial. Afterwards, two rearing densities (low density and high density, LD and HD, respectively) were established by randomly distributing 40 and 120 fish per tank (96.2 \pm 2.1g) in six 800L tanks 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 (overall water volume: 15 m^{-3}). The rearing system consisted of a mechanical sand filter 148 (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25mJ cm⁻²: 32 m⁻³ h⁻¹, 149 150 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 ± 1.0 °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). Ammonia (total ammonia nitrogen $\leq 0.1 \text{ mg } \text{L}^{-1}$) and nitrite ($\leq 0.2 \text{ mg } \text{L}^{-1}$) were daily 156 157 monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany) while salinity (30 g L^{-1}) was measured by a salt refractometer (106 158 159 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 anaesthetised by 2-phenoxyethanol at 300 mg L^{-1} and individually weighed. The 169 170 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

then obtained by pooling the 3 fish sampled per tank during the analyses, as the tank was considered as the experimental unit and not the organism. At the same time, digesta content from posterior intestine (n = 15 fish per diet treatment, n = 5 fish per replicate) was also individually sampled and immediately stored at -80 °C for gut microbiota 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 x g, 10 min, 4°C) and plasma aliquots were stored at -80 °C until analysis (Bonvini et al., 193 194 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.

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

203 2.4 Calculations

204

The following formulae were used to calculate different performance parameters: specific growth rate (SGR) (% day⁻¹) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights, respectively). Feed Intake (FI) (g kg $ABW^{-1} day^{-1}$)=((1000 * total ingestion)/(ABW))/days)) (where average body weight, 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

sample deterioration. In brief, the stomach and pyloric caeca samples (including 1 cm of anterior intestine) were homogenized in 5 volumes (ww/v) of distilled water at 4 °C for 1 min followed by a sonication process of 30 sec. After a centrifugation (9,000 x *g* for 10 min at 4 °C), the supernatant was collected, aliquoted and stored at -20° C for the 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) pH 7.0, at a maximum speed for 30 s (IKA, Ultra-turrax[®], USA), then 100 µL of 0.1M 241 242 CaCl₂ was added to the homogenate, stirred and centrifuged (9,000 x g for 10 min at 4 °C). A fraction of the supernatant was collected and stored at -20 °C for the leucine-243 244 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 Tris-HCl 50 nmol L^{-1} (pH = 9). One unit (U) of activity was defined as the nmoles of azo 249 250 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 hydrolyzed min⁻¹ mL⁻¹ of enzyme extract at $\lambda = 407$ nm (Holm et al., 1988). 253 254 Chymotrypsin activity was quantified using BTEE (benzoyl tyrosine ethyl ester) as 255 substrate and its activity (U) corresponded to the μ mol BTEE hydrolyzed min⁻¹ mL⁻¹ of 256 enzyme extract at $\lambda = 256$ 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 x 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 µmol of pNP released min⁻¹ mL⁻¹ of brush border homogenate at $\lambda = 407$ nm (Gisbert et al., 2018). 268 269 Aminopeptidase-N was determined using 80 mM 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 µg 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 (U) was defined as 1 nmol of the hydrolyzed substrate $min^{-1} mL^{-1}$ of tissue homogenate 278 279 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 25-26 °C and expressed as specific activity defined as units per mg of protein (U mg protein⁻¹). All the assays were made in triplicate (methodological replicates) for each tank and the absorbance was read using a 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 μ L 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 $\lambda = 450$ 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 $U m L^{-1}$ equivalent of HEWL activity.

Protease activity was quantified using the azocasein hydrolysis assay according to Guardiola et al. (2014). Aliquots of 100 μ L of each mucus sample were incubated with 100 μ L of 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma-Aldrich) for 19 h at 30 °C. The reaction was stopped by adding 4.6% trichloro acetic acid (TCA) and the mixture centrifuged (10,000 x g, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 μ L well⁻¹ of 0.5 N NaOH. In both cases, the OD was read at $\lambda = 450$ 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) with the same volume of standard trypsin solution (5 mg mL⁻¹) in a 96-well flat-bottomed 310 311 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 $(10,000 \times g, 10 \text{ min})$ and the supernatant was transferred to a 96-well plate in triplicate, containing 100 µL well⁻¹ of 1 N NaOH before 315 316 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. Briefly, 2 mg mL⁻¹ solution of BSA was prepared and serial dilutions made with 323 324 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 mucus. All spectrophotometry reads were conducted with a Varioskan 2.4.5, (ThermoScientific, 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).

354 2.9 Metabolic parameters in plasma

355

356 The levels of glucose (GLU), urea, creatine, uric acid, total bilirubin, bile acid, amylase, lipase, cholesterol (CHOL), triglycerides (TRIG), total protein (TP), albumin 357 358 (ALB), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline 359 phosphatase (ALP), gamma-glutamyl transferase (GGT), creatine kinase (CK), lactate dehydrogenase (LDH), calcium (Ca⁺²), phosphorus (P), potassium (K⁺) sodium (Na⁺), 360 361 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 of 500 µL on an automated analyser (AU 400; Beckman Coulter) according to the 363 364 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 ($p \le 0.05$) Tukey's post hoc test was 376 performed. The normality and/or homogeneity of variance assumptions were validated

for all data preceding ANOVA. The R packages "Stats" and "Vegan" were used to 377 378 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 performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, 384 385 USA) and RStudio interface for R (https://www.r-project.org). The differences among 386 treatments were considered significant at $p \le 0.05$.

- 387
- 388 **3. Results**
- 389

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 then 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 > 0.05). 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).

³⁹⁰ *3.1 Growth*

401Data on body composition and nutritional indices are shown in Table 3. Whole body402composition values were not significantly influenced by different fish density (p > 0.05),403while lipid content was lower in fish fed the FM10/FO3 diet compared to the FM30/FO15404group (p < 0.05); however, ash and moisture levels were higher in FM10/FO3 than405FM30/FO15 fish (p < 0.05). No significant effects of fish density on PER, GPE, GLE and406LER were detected (p > 0.05); however, fish fed FM10/FO3 displayed lower PER, GPE,407GLE 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 (p420 < 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 ($p > 0.05$).

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 significant effect of rearing density was detected (p > 0.05). Specifically, protease activity in skin mucus was significantly higher in fish fed the FM30/FO15 diet at both rearing densities compared to those fed FM10/FO3 and reared at LD (Fig 1B; p < 0.05). No significant effect of density or diet were detected in antiprotease activity and total proteins of skin mucus from fish belonging to the different experimental groups (Fig. 1, C-D; p >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% ± 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% ± 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 $(14.5-36/44 \text{ kg m}^{-3}, \text{initial})$ 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 $(4.8-12/15 \text{ kg m}^{-3})$. The results of the present study during the on-growing phase (96-318g) go beyond the maximum density tested (20-31 kg m⁻³) by Araújo-Luna et al. 508 509 (2018) for gilthead sea bream at similar size (268-435 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 (22-85 g) in which high density up to 40.8 kg m^{-3} did not negatively affect growth (Montero et al., 513 514 1999). However, more recently, Diogenes et al. (2019) found that rearing density up to 57 kg m^{-3} impaired FI, growth and FCR in sea bream juveniles (12-58g). The authors 515 suggested that 40 kg m^{-3} could be near the maximum tolerable stocking density for 516 517 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 FM-based diet at low rearing density (3-5 kg m^{-3}) and in line with those found by 586 587 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.,

592 1999; Lalles et al., 2019). The values of plasma electrolytes provided in the trial were 593 comparable with the values reported in sea bream (Peres et al., 2013; Guardiola et al., 594 2018) and sobaity sea bream (Sparidentex hasta) (Hekmatpoure et al., 2019). Plasma 595 electrolytes are indicators of the secondary phase of stress response in fish, providing an 596 indirect indication of altered plasma cortisol levels; in particular plasma phosphorus and 597 calcium levels were found to be sensitive to fish stocking density (Hrubec et al., 2000) 598 while potassium levels are accepted as a general indicator of stress in fish (Guardiola et 599 al., 2018).

600 Evaluation of skin mucosal immunity has been proposed recently as a promising 601 alternative stress assessment in fish species after stressful conditions including crowding 602 or transportation, whereas data of specific mucosal component in response to different 603 stressors are still scarce (Guardiola et al 2016; Sanahuja et al., 2019). Enzymes in the 604 epidermal mucus such as lysozyme, protease and antiprotease play an important role in 605 humoral and skin mucus defence acting directly on a pathogen, or activating and 606 enhancing the production of various immunological components of fish subjected to 607 stressful situations (Esteban, 2012; Guardiola et al., 2016). The present results indicate 608 different effects of treatments on specific skin mucus components, lysozyme being 609 slightly reduced by high rearing density while protease was mainly reduced by low FM-610 FO diets. Both enzymes have been shown to be modulated either by diet or environmental 611 conditions in sea bream. Most studies have shown the possibility of increasing lysozyme 612 activity of skin mucus by dietary additives, such as selenium nanoparticles, Moringa 613 *oleifera* leaves or probiotics; but crowding conditions at 20 kg m⁻³ for 30 days has also 614 been reported to lead to an increase in lysozyme gene expression in sea bream skin mucus 615 (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 48 h of acute 50 kg m⁻³ crowding stress. However, in the same study a reduction in the 617 618 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 Streptococcacee abundances compared to low FM-FO diet, while under low rearing 671 672 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.

Analysis of biodiversity of the microbial community has highlighted a different response to the feeding regimes, showing a general higher biodiversity in fish fed diets containing higher vegetable ingredients. This is in general agreement with previous findings detecting feeding habit as a key factor influencing fish gut microbial diversity and observing an increasing trend in diversity following the order of carnivores, omnivores and herbivores (Wang et al., 2018). In addition, a significant increase in α 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 density up to 36-44 kg m⁻³ and that a high level of vegetable dietary ingredients does not 732 733 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 gilthead sea bream at the on-growing phase at a density up to $36-44 \text{ kg m}^{-3}$ with low or 742 743 high FM-FO diet without negatively affecting growth, feed efficiency, welfare condition 744 and gut microbial community.

745

746 Acknowledgment

747

748 This research was supported by ERC (European Research Council) in MedAID project 749 (Mediterranean Aquaculture Integrated Development), Call H2020-SFS-2016-2017 750 (Sustainable Food Security - Resilient and resource-efficient value chains), Grant 751 Agreement n. 727315. Analyses of digestive enzymes conducted at IRTA were partially 752 supported by the project ADIPOQUIZ (RTI2018-095653-R-I00) funded by the 753 Ministerio de Ciencia, Innovación y Universidades (Spain). The authors would like to 754 thank Gillian Forlivesi Heywood for English language editing and Stefano Porcelli for 755 the technical contribution in fish rearing and laboratory analysis.

- 757
- 758
- 759

760 **References**

- 761
- Alves, R.N., Cordeiro, O., Silva, T.S., Richard, N., de Vareilles, M., Marino, G., Di
 Marco, P., Rodrigues, P.M., Conceição, L.E.C., 2010. Metabolic molecular
 indicators of chronic stress in gilthead seabream (*Sparus aurata*) using
 comparative proteomics. Aquaculture, 299, 1-4.
- 766 Apper, E., Weissman, D., Respondek, F., Guyonvarch, A., Baron, F., Boisot, P., Rodiles,
- A., Merrifield, D.L., 2016. Hydrolysed wheat gluten as part of a diet based on
 animal and plant proteins supports good growth performance of Asian seabass
 (*Lates calcarifer*), without impairing intestinal morphology or microbiota.
 Aquaculture 453, 40–48.
- Araújo-Luna, R., Ribeiro, L., Bergheim, A., Pousão-Ferreira, P., 2018. The impact of
 different rearing condition on gilthead seabream welfare: Dissolved oxygen levels
 and stocking densities. Aquac. Res. 49 (12).
- Ashley, P.J., 2007. Fish welfare: current issues in aquaculture. Appl. Anim. Behav. Sci.
 104, 199–235.
- Azeredo, R., Machado, M., Martos-Sitcha, J.A., Martínez-Rodríguez, G., Moura, J.,
 Peres, H., Oliva-Teles, A., Afonso, A., Mancera, J.M., Costas, B. 2019. Dietary
 tryptophan induces opposite health-related responses in the Senegalese sole (*Solea senegalensis*) reared at low or high stocking densities with Implications in disease
 resistance. Front. Physiol. art. no. 508.
- Bailey, M.T., Dowd, S.E., Galley, J.D., Hufnagle, A.R., Allen, R.G., Lyte, M., 2011.
 Exposure to a social stressor alters the structure of the intestinal microbiota:

- 783 Implications for stressor-induced immunomodulation. Brain Behav. Immun. 25784 (3), 397-407.
- 785 Biagi, E., D'Amico, F., Soverini, M., Angelini, V., Barone, M., Turroni, S., Rampelli, S.,
- Pari, S., Brigidi, P., Candela, M., 2018. Faecal bacterial communities from
 Mediterranean loggerhead sea turtles (*Caretta caretta*). Environ Microbiol Rep.
 doi: 10.1111/1758-2229.12683.
- Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification.
 Can. J. Biochem. Phys. 37, 911–917.
- 791 Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A.,
- Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al., 2019. Reproducible,
 interactive, scalable and extensible microbiome data science using QIIME 2. Nat.
 Biotechnol. 37 (8), 852-857.
- Bonga, S.E.W., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.

796 Bonvini, E., Bonaldo, A., Parma, L., Mandrioli, L., Sirri, R., Grandi, M., Fontanillas, R.,

- Viroli, C., Gatta, P.P., 2018a. Feeding European sea bass with increasing dietary
 fibre levels: Impact on growth, blood biochemistry, gut histology, gut evacuation,
 Aquaculture, 494, 1-9.
- Bonvini, E., Bonaldo, A., Mandrioli, L., Sirri, R., Dondi, F., Bianco, C., Fontanillas, R.,
 Mongile, F., Gatta, P.P., Parma, L., 2018b. Effects of feeding low fishmeal diets
 with increasing soybean meal levels on growth, gut histology and plasma
 biochemistry of sea bass. Animal, 12(5), 923-930.
- Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram
 quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.
 72, 248–254.

808	2016. DADA2: high-resolution sample inference from Illumina amplicon data.
809	Nat Methods 13, 581–583.
810	Canario, A.V.M., Condeça, J., Power, D.M., Ingleton, P.M., 1998. The effect of
811	stockingdensity on growth in the gilthead sea-bream, Sparus aurata (L.). Aquac.
812	Res. 29, 177–181.
813	Carda-Diéguez, M., Mira, A., Fouz, B., 2014. Pyrosequencing survey of intestinal
814	microbiota diversity in cultured sea bass (Dicentrarchus labrax) fed functional
815	diets. Fems Microbiol. Ecol. 87, 451–459.
816	Chao, A., N. J. Gotelli, T. Hsieh, E. L. Sander, K. Ma, R. K. Colwell, and A. M. Ellison.
817	2014. Rarefaction and extrapolation with Hill numbers: a framework for sampling
818	and estimation in species diversity studies. Ecological Monographs 84, 45–67.
819	Cordero, H., Morcillo, P., Cuesta, A., Brinchmann, M.F., Esteban, M.A., 2016.
820	Differential proteome profile of skin mucus of gilthead seabream (Sparus aurata)
821	after probiotic intake and/or overcrowding stress. J. Proteomics 132, 41-50.
822	Dahkqvist, A., 1970. Assay of intestinal disaccharidase. Enzym. Biol. Clin. 11, 52-66.
823	Dawood, M.A.O., Koshio, S., Zaineldin, A.I., Van Doan, H., Moustafa, E.M., Abdel-
824	Daim, M.M., Angeles Esteban, M., Hassaan, M.S., 2019. Dietary supplementation
825	of selenium nanoparticles modulated systemic and mucosal immune status and
826	stress resistance of red sea bream (Pagrus major). Fish Physiol. Biochem., 45 (1),
827	219-230.
828	De las Heras, V., Martos-Sitcha, J.A., Yúfera, M., Mancera, J.M., Martínez-Rodríguez,
829	G., 2015. Influence of stocking density on growth, metabolism and stress of
830	thicklipped grey mullet (Chelon labrosus) juveniles. Aquaculture 448, 29-37.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P.,

- Balancey, K., Agius, C., 2003. Enzyme activities and pH variations in the
 digestive tract of gilthead sea bream. J. Fish Biol., 62(5), 1033-1043.
- 833 Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G.,
- Hill, J.E., 2012. Effects of plant-based diets on the distal gut microbiome of
 rainbow trout (*Oncorhynchus mykiss*). Aquaculture 350, 134-142.
- 836 Diógenes, A.F., Teixeira, C., Almeida, E., Skrzynska, A., Costas, B., Oliva-Teles, A.,
- Peres, H., 2019. Effects of dietary tryptophan and chronic stress in gilthead
 seabream (*Sparus aurata*) juveniles fed corn distillers dried grains with solubles
 (DDGS) based diets. Aquaculture 498, 396-404.
- B40 Du, F., Li, Y., Tang, Y., Su, S., Yu, J., Yu, F., Li, J., Li, H., Wang, M., Xu, P., 2019.
 Response of the gut microbiome of *Megalobrama amblycephala* to crowding
 stress. Aquaculture 500, 586-596.
- Easy, R.H., Ross, N.W., 2010. Changes in Atlantic salmon *Salmo salar* mucus
 components following short- and long-term handling stress. J. Fish Biol, 77, 16161631.
- Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. Review
 paper: the relationships between stocking density and welfare in farmed rainbow
 trout. J. Fish. Biol. 61:493–531.
- Esteban, M. A., 2012. An overview of the immunological defenses in fish skin, ISRN
 Immunol. 1–29 <u>https://doi.org/10.5402/2012/853470</u>.
- 851 Estruch, G., Collado, M.C., Peñaranda, D.S., Tomás Vidal, A., Jover Cerdá, M., Pérez
- 852 Martínez, G., Martinez-Llorens, S., 2015. Impact of fishmeal replacement in diets
- determined by pyrosequencing the 16S rRNA gene. PLoS One 10, e0136389.

for gilthead sea bream Sparus aurata on the gastrointestinal microbiota

- Gajardo, K., Jaramillo-Torres, A., Kortner, T.M., Merrifield, D.L., Tinsley, J., Bakke,
 A.M., Krogdahl, Å., 2017. Alternative protein sources in the diet modulate
 microbiota and functionality in the distal intestine of Atlantic salmon (*Salmo salar*). Appl. Environ. Microbiol. 83, e02615.
- Galley, J.D., Nelson, M.C., Yu, Z., Dowd, S.E., Walter, J., Kumar, P.S., Lyte, M., Bailey,
 M.T., 2014. Exposure to a social stressor disrupts the community structure of the
 colonic mucosa-associated microbiota. BMC Microbiol. 14, 189.
- Gatesoupe, F.J., Huelvan, C., Le Bayon, N., Le Delliou, H., Madec, L., Mouchel, O.,
 Quazuguel, P., Mazurais, D., Zambonino-Infante, J., 2016. The highly variable
 microbiota associated to intestinal mucosa correlates with growth and hypoxia
 resistance of sea bass, *Dicentrarchus labrax*, submitted to different nutritional
 histories. BMC Microbiol. 16, 266.
- Gisbert, E., Gimenéz, G., Fernández, I., Kotzamanis, Y., Estevéz, A., 2009. Development
 of digestive enzymes in common dentex *Dentex dentex* during early ontogeny.
 Aquaculture 287, 381–387.
- Gisbert, E., Nolasco, H., Solovyev, M., 2018. Towards the standardization of brush
 border purification and intestinal alkaline phosphatase quantification in fish with
 notes on other digestive enzymes. Aquaculture 487, 102-108.
- Guardiola, F.A., Cuesta, A., Arizcun, M., Meseguer, J., Esteban, M.A., 2014.
 Comparative skin mucus and serum humoral defence mechanisms in the teleost
 gilthead seabream (*Sparus aurata*). Fish Shellfish Immunol. 36, 545–551.
- Guardiola, F.A., Cuesta, A., Esteban, M.A., 2016. Using skin mucus to evaluate stress in
 gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 59, 323-330.

- Guardiola, F.A., Saraiva-Fraga, M., Cuesta, A., Esteban, M.A., 2018. Changes in natural
 haemolytic complement activity induced by stress in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 78, 317-321.
- Hanif, A., Bakopoulos, V., Dimitriadis, G.J., 2004. Maternal transfer of humoral specific
 and non-specific immune parameters to seabream (*Sparus aurata*) larvae. Fish
 Shellfish Immunol, 17, 411-435.
- Hekmatpour, F., Kochanian, P., Marammazi, J.G., Zakeri, M., Mousavi, S.-M., 2019.
 Changes in serum biochemical parameters and digestive enzyme activity of
 juvenile sobaity sea bream (*Sparidentex hasta*) in response to partial replacement
 of dietary fish meal with poultry by-product meal. Fish Physiol. Biochem. 45,
 599-611.
- Hill, M. 1973. Diversity and evenness: A unifying notation and its consequences. Ecology
 54, 427–432.
- Holm, H., Hanssen, L.E., Krogdahl, A., Florholmen, J., 1988. High and low inhibitor
 soybean meals affect human duodenal proteinase activity differently: in vivo
 comparison with bovine serum albumin. J. Nutr. 118, 515–520.
- Hrubec, T. C., Cardinale, J. L., Smith, S. A., 2000. Hematology and plasma chemistry
 reference intervals for cultured tilapia (Oreochromis hybrid). Vet. Clin. Pathol.
 29, 7–12.
- 897 Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile-salt
 898 activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish
 899 Physiol. Biochem. 18, 59–69.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O.,
 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical

and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41

903 (1), e1, <u>http://dx.doi.org/10.1093/nar/gks808</u>.

- Lallès, J.P., 2019. Intestinal alkaline phosphatase in the gastrointestinal tract of fish:
 biology, ontogeny, and environmental and nutritional modulation. Rev. Aquac.
 DOI: 10.1111/raq.12340.
- Li, L., Chen, S., Li, X., Wan, D., Liu, G., Yin, Y. L., 2017. Intestinal microbiota in
 growing pigs: Effects of stocking density. Food Agric. Immunol.
 doi:10.1080/09540105.2017 1409195
- Lemieux, H., Blier, P., Dutil, J.D., 1999. Do digestive enzymes set a physiological limit
 on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*)? Fish Physiol. Biochemist 20, 293–303.
- Mancera, J.M., Vargas-Chacoff, L., García-López, A., Kleszczyńska, A., Kalamarz, H.,
 Martínez-Rodríguez, G., Kulczykowska, E., 2008. High density and food
 deprivation affect arginine vasotocin, isotocin and melatonin in gilthead sea bream
- 916 (Sparus auratus). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 149, 92–97.
- 917 Mansour, A.T., Miao, L., Espinosa, C., García-Beltrán, J.M., Ceballos Francisco, D.C.,
- Esteban, M.Á., 2018. Effects of dietary inclusion of *Moringa oleifera* leaves on
 growth and some systemic and mucosal immune parameters of seabream. Fish
 Physiol. Biochemist. 44 (4), 1223-1240.
- Maroux, S., Louvard, D., Baratti, J., 1973. The aminopeptidase from hog-intestinal brush
 border. Biochim. Biophys. Acta 321, 282–295.
- Messina, M., Bulfon, C., Beraldo, P., Tibaldi, E., Cardinaletti, G., 2019. Intestinal
 morpho-physiology and innate immune status of European sea bass

- 925 (Dicentrarchus labrax) in response to diets including a blend of two marine 926 microalgae, *Tisochrysis lutea* and *Tetraselmis suecica*. Aquaculture 500, 660-669. 927 Métais, P., Bieth, J., 1968. Détermination de l'a-amylase par une microtechnique. Ann. 928 Biol. Clin. 26, 133–142. 929 Miao, S., Zhao, C., Zhu, J., Hu, J., Dong, X., Sun, L., 2018. Dietary soybean meal affects 930 intestinal homoeostasis by altering the microbiota, morphology and inflammatory 931 cytokine gene expression in northern snakehead. Sci. Rep. 8, 113. 932 https://doi.org/10. 1038/s41598-017-18430-7. 933 Millán-Cubillo, A. F., Martos-Sitcha, J. A., Ruiz-Jarabo, I., Cárdenas, S., Mancera, J. M., 934 2016. Low stocking density negatively affects growth, metabolism and stress 935 pathways in juvenile specimens of meagre (Argyrosomus regius, Asso 1801). 936 Aquaculture 451, 87–92.
- Mommsen, T. P., Vijayan, M. M., Moon, T. W., 1999. Cortisol in teleosts: dynamics,
 mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fisher 9, 211–
 268. doi: 10.1023/A:1008924418720.
- Montero, D., Izquierdo, M.S., Tort, L., Robaina, L., Vergara, J.M., 1999. High stocking
 density produces crowding stress altering some physiological and biochemical
 parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol.
 Biochem. 20, 53–60.
- Nicholson, J.A., Kim, Y.S., 1975. A one-step L-amino acid oxidase assay for intestinal
 peptide hydrolase activity. Anal. Biochem. 63, 110–117.
- 946 Parma, L., Candela, M., Soverini, M., Turroni, S., Consolandi, C., Brigidi, P., Mandrioli,
- 947 L., Sirri, R., Fontanillas, R., Gatta, P.P., Bonaldo, A., 2016. Next-generation
 948 sequencing characterization of the gut bacterial community of gilthead sea bream

- 949 (*Sparus aurata*, L.) fed low fishmeal based diets with increasing soybean meal
 950 levels. Anim. Feed Sci. Technol. 222, 204–216.
- Parma, L., Yúfera, M., Navarro-Guillén, C., Moyano, F.J., Soverini, M., D'Amico, F.,
 Candela, M., Fontanillas, R., Gatta, P.P., Bonaldo, A., 2019. 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. Aquaculture, 510, 283-292.
- Peres, H., Santos, S., Oliva-Teles, A., 2013. Selected plasma biochemistry parameters in
 gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 29, 630–636.
- Piazzon, M.C., Calduch-Giner, J.A., Fouz, B., Estensoro, I., Simó-Mirabet, P., Puyalto,
 M., Karalazos, V., Palenzuela, O., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2017.
 Under control: how a dietary additive can restore the gut microbiome and
 proteomic profile, and improve disease resilience in a marine teleostean fish fed
 vegetable diets. Microbiome 5:Article 164 DOI 10.1186/s40168-017-0390-3.
- 963 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J.,
 964 Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project:
 965 improved data processing and web-based tools. Nucleic Acid Res. 41, 590-596.
- Ran, C., Huang, L., Hu, J., Tacon, P., He, S., Li, Z., Wang, Y., Liu, Z., Xu, L., Yang, Y.,
 Zhou, Z., 2016. Effects of dietary live and heat-inactive baker's yeast on growth,
 gut health, and disease resistance of Nile tilapia under high rearing density. Fish
 Shellfish Immunol. 56, 263-271.
- 870 Rimoldi, S., Gliozheni, E., Ascione, C., Gini, E., Terova, G., 2018a. Effect of a specific
 871 composition of short- and medium-chain fatty acid 1-Monoglycerides on growth

- 972 performances and gut microbiota of gilthead sea bream (*Sparus aurata*). PeerJ,
 973 (7), art. no. e5355.
- 874 Rimoldi, S., Terova, G., Ascione, C., Giannico, R., Brambilla, F., 2018b. Next generation
 875 sequencing for gut microbiome characterization in rainbow trout (*Oncorhynchus*876 *mykiss*) fed animal by-product meals as an alternative to fish meal protein sources.
- 977 PLoS One 13, e0193652. https://doi.org/10.1371/journal.pone.0193652.
- 8 Ringo, E., Hossein, S., Ghosh, K., Doan, H.V., Beck, B.R., Song, S., 2018. Lactic acid
 bacteria in finfish–an update. Front. Microbiol. 9, 1818.
- Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: A versatile
 open source tool for metagenomics. PeerJ, 10, art. no. e2584.
- Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Sánchez-Nuño, S., Ramos, A.,
 Araujo, R.M., Ibarz, A., 2019. Comparison of several non-specific skin mucus
 immune defences in three piscine species of aquaculture interest. Fish Shellfish
 Immunol. 89, 428-436.
- 986 Sánchez-Muros, M.J., Sánchez, B., Barroso, F.G., Toniolo, M., Trenzado, C.E., Sanz Rus,
- A., 2017. Effects of rearing conditions on behavioural responses, social kinetics
 and physiological parameters in gilthead sea bream *Sparus aurata*. Appl. Anim.
 Behav. Sci. 197, 120-128.
- Sangiao-Alvarellos, S., Guzmán, J.M., Laiz-Carrión, R., Míguez, J.M., Martín del Río,
 M.P., Mancera, J.M., Soengas, J.L., 2005. Interactive effects of high stocking
 density and food deprivation on carbohydrate metabolism in several tissues of
 gilthead sea bream Sparus auratus. J. Exp. Zool. A. 303, 761–775.

- Singh, A., Faber-Hammond, J.J., O'Rourke, C.F., Renn, S.C.P., 2019. Gut microbial
 diversity increases with social rank in the African cichlid fish, Astatotilapia
 burtoni, Anim Behav 152, 79-91.
- 997 Skrzynska, A.K., Martos-Sitcha, J.A., Martínez-Rodríguez, G., Mancera, J.M., 2018.
 998 Unraveling vasotocinergic, isotocinergic and stress pathways after food
 999 deprivation and high stocking density in the gilthead sea bream. Comp. Biochem.
 1000 Physiol. A-Mol. Integr. Physiol. 215, 35-44.
- Solovyev, M., Gisbert, E., 2016. Influence of time, storage temperature and
 freeze/thawcycles on the activity of digestive enzymes from gilthead sea bream
 (*Sparus aurata*). Fish Physiol. Biochem. 42, 1383–1394.
- Soverini, M., Quercia, S., Biancani, B., Furlati, S., Turroni, S., Biagi, E., Consolandi, C.,
 Peano, C., Severgnini, M., Rampelli, S., Brigidi, P., Candela, M., 2016. The
 bottlenose dolphin (*Tursiops truncatus*) faecal microbiota. FEMS Microbiol Ecol.
 92, (4).
- 1008Stothart, M. R., Bobbie, C. B., Schulte-Hostedde, A. I., Boonstra, R., Palme, R.,1009Mykytczuk, N. C. S., et al., 2016. Stress and the microbiome: Linking1010glucocorticoids to bacterial community dynamics in wild red squirrels. Biol. Lett.
- 1011 12(1), 20150875. https://doi.org/10.1098/rsbl.2015.0875.
- 1012 Swain, N.S.P., Dash, S., Sahoo, P.K., Routray, P., Sahoo, S.K., Gupta, S.D., Meher, P.K.,
- 2007. Non-specific immune parameters of brood Indian major carp *Labeo rohita*and their seasonal variations. Fish Shellfish Immunol. 22, 38–43.
- 1015 Tort, L., 2011. Stress and immune modulation in fish. Dev. Comp. Immunol. 35, 1366–1016 1375.

1018	Sanz, A., 2018. Effect of dietary lipid content and stocking density on digestive
1019	enzymes profile and intestinal histology of rainbow trout (Oncorhynchus mykiss),
1020	Aquaculture, 497, 10-16.
1021	Wang, A.n R., Ran, C., Ringø, E., Zhou, Z., 2018. Progress in fish gastrointestinal
1022	microbiota research. Rev. Aquacult. 10, 626–640.
1023	Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch,
1024	T., Wiens, G.D., Snekvi, K., Rawls, J., Good, C., 2013. Aquacultured rainbow
1025	trout (Oncorhynchus mykiss) possess a large core intestinal microbiota that is
1026	resistant to variation in diet and rearing density. Appl. Environ. Microbiol. 79
1027	(16), 4974-4984.
1028	Worthington Biochemical Corporation, 1991. Worthington Enzyme Manual: Enzymes,
1029	Enzyme Reagents, Related Biochemical. Worthington Biochemical Corp.,
1030	Freehold, New Jersey.
1031	Zha, Y., Eiler, A., Johansson, F., Svanbäck, R., 2018. Effects of predation stress and food
1032	ration on perch gut microbiota. Microbiome, 6 (1), art. no. 28.
1033	
1034	
1035	
1036	
1037	
1038	
1039	
1040	

Trenzado, C.E., Carmona, R., Merino, R., García-Gallego, M., Furné, M. Domezain, A.

1017

Table 1. Ingredients and proximate composition of the experimental dietsFM30/F015FM10/F03Ingredients, % of the dietFish meal (LT70)30.010.0Soybean meal 489.09.0Soy protein concentrate10.020.5Wheat gluten5.010.2Computer protein concentrate10.015.0

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
Proximate composition, % on a wet weigh	t basis	
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

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								
					P value			
	FM30	/FO15	FM10	Density	Diet	Inter		
	LD	HD	LD	HD				
Initial density kg m ⁻³	4.8±0.1ª	14.5±0.6 ^b	4.8±0.1ª	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±1.3ª	35.9±0.5°	<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±25.3ª	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±0.21ª	15.4 ± 0.64^{ab}	14.5±0.03ª	0.002	0.506	0.818	
FCR	1.43 ± 0.02^{a}	1.42±0.01ª	1.70±0.21 ^b	1.61 ± 0.02^{ab}	0.433	0.005	0.495	
Survival %	$95.8{\pm}1.4^{a}$	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 $(n=3) \pm SD$. In each line, different superscript letters indicate significant differences among

treatments ($P \le 0.05$). 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)

1048

-	Experimental diets						
	FM30/FO15		FM10/FO3		P-value		
	LD	HD	LD	HD	Density	Diet	Inter.
Whole body composition, %	1						
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}	$16.6\pm0.7^{\rm a}$	$17.0\pm0.8^{\rm a}$	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}	$91.7\pm9.0^{\rm b}$	$60.9\pm9.4^{\rm a}$	$66.2\pm4.6^{\rm a}$	0.768	0.000	0.253
LER	$4.08\pm0.05^{\text{b}}$	4.11 ± 0.03^{b}	$3.32\pm0.40^{\rm a}$	$3.48\pm0.04^{\rm a}$	0.476	0.000	0.579

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.

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

1050

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.

	Experimental diets						
	FM30	/FO15	FM10/	/FO3	<i>P-value</i>		
	LD	HD	LD	HD	Density	Diet	Inter.
Pancreatic (Stomach/AI)							
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	$0.01\ 7 \pm 0.01$	0.017 ± 0.01	0.022 ± 0.02	2 ± 0.02 0.025 ± 0.01		0.264	0.819
Brush border AI							
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	$33.0{\pm}3.1^{ab}$	$62.3\pm18.7^{\text{b}}$	24.7 ± 6.8^a	41.3 ± 4.8^{ab}	0.011	0.065	0.374
Brush Border PI							
Aminopeptidase	$0.043\pm0.01^{\text{b}}$	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}	$41.8\pm0.9^{\rm a}$	0.038	0.430	0.058

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

<u>^</u>	Experimental diets						
-	FM30/	FO15	FM10)/FO3			
Parameters	LD	HD	LD	HD	Density	Diet	Interaction
Glucose (mg dL ⁻¹)	119±26	123±29	117±31	101±24	0.374	0.079	0.145
Urea (mg dL ⁻¹) Creatine (mg dL ⁻¹)	${}^{10.7\pm2.0^{ab}}_{0.37\pm0.14^{b}}$	$\begin{array}{c} 9.25{\pm}1.44^{a} \\ 0.30{\pm}0.10^{b} \end{array}$	${}^{11.6\pm2.1^{bc}}_{0.22\pm0.04^a}$	$\begin{array}{c} 13.5{\pm}2.8^{c}\\ 0.21{\pm}0.04^{a} \end{array}$	0.760 0.169	$0.000 \\ 0.000$	0.003 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 d L^{-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. (μ mol 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±0.41ª	0.213	0.001	0.909
ALB (g dL ^{-1})	0.97 ± 0.19^{b}	$0.90{\pm}0.15^{ab}$	0.89 ± 0.06^{ab}	$0.84{\pm}0.10^{a}$	0.081	0.040	0.724
$AST(UL^{-1})$	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
$\operatorname{Ca}^{+2}(\operatorname{mg} \operatorname{dL}^{-1})$	$15.0{\pm}1.7^{b}$	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	$12.0{\pm}1.8$	12.2 ± 1.4	12.3 ± 2.4	0.249	0.381	0.183
K^+ (mEq L^{-1})	7.16 ± 2.45^{b}	$5.28{\pm}1.58^{a}$	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 ($\mu g dL^{-1}$)	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 ($\mu g dL^{-1}$)	464±78	433±97	502±68	488±96	0.300	0.031	0.695
TIBC ($\mu g dL^{-1}$)	599±97	544±116	626±74	616±105	0.193	0.049	0.373
Cortisol ($\mu g dL^{-1}$)	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}	$0.28{\pm}0.02^{a}$	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

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

Data are given as the mean $(n=15) \pm SD$. Different letters indicate significant difference ($P \le 0.05$) 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.

1	062
1	063
1	064
1	065

1067 Key to Figures

Figure 1. A, Lysozyme (U mL⁻¹); B, protease activity (%); C, antiprotease activity (%); D, total protein (mg mL⁻¹) in skin mucus of gilthead seabream reared at low (LD, light grey) and high (HD, dark grey) stocking density and fed the experimental diets over 98 days. FM30/FO15 = 300g kg⁻¹ fishmeal (FM), 150 g kg⁻¹ fish oil (FO); FM10/FO3 = 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).

Figure 2. Barplots representing the sea bream gut bacterial community at two phylogenetic levels: A) phylum; B) Family. In panel C) are reported the boxplots with the families showing a significant difference in relative abundance among groups (p value < 0.05, Wilcoxon ran-sum test; FDR correction). 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.

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; Wilconxon 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}, p > 0.05; permutation test with pseudo-F ratios, Adonis). FM30/FO15 = 300g kg⁻¹ 1085 fishmeal (FM), 150 g kg⁻¹ fish oil (FO); FM10/FO3 = $100g kg^{-1}$ FM; $30g kg^{-1}$ FO. LD, 1086 1087 low rearing density; HD, high rearing density.















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/F0	015 _{hd}	FM30/F0	D15 LD	FM10/F	O3 _{HD}	FM10/I	FO3 _{LD}
Phylum	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Actinobacteria	6.7	6.0	5.0	3.0	12.5	7.1	7.8	8.9
Bacteroidetes	1.4	4.3	0.2	0.3	0.5	0.4	0.9	0.7
Chlamydiae	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
Chloroflexi	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
Cyanobacteria	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5
Firmicutes	87.3	9.4	92.2	4.3	69.9	13.4	77.9	13.7
Gracilibacteria	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
Lentisphaerae	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Planctomycetes	0.1	0.3	0.3	0.2	0.8	0.9	1.4	1.7
Proteobacteria	2.5	2.9	1.2	0.9	7.6	6.3	7.1	6.1
Saccharibacteria	0.3	0.5	0.2	0.4	0.6	1.3	0.8	1.1
Spirochaetae	0.1	0.4	0.0	0.0	0.5	1.7	0.0	0.0
TM6 (Dependentiae)	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.2
Verrucomicrobia	0.0	0.1	0.0	0.2	0.1	0.3	0.0	0.0
WS6	0.0	0.0	0.0	0.1	0.2	0.7	0.0	0.1
Unassigned;Other	0.0	0.1	0.1	0.2	0.2	0.8	0.2	0.4
Class								
Acidimicrobiia	0.0	0.0	0.1	0.1	0.5	0.9	0.9	1.6
Actinobacteria	6.0	5.6	4.9	3.0	11.6	7.1	6.7	7.7
Coriobacteriia	0.4	0.9	0.0	0.1	0.4	0.8	0.1	0.3
Thermoleophilia	0.3	1.0	0.0	0.0	0.1	0.2	0.0	0.0
Bacteroidia	1.3	4.3	0.1	0.2	0.1	0.1	0.4	0.5
Flavobacteriia	0.1	0.2	0.1	0.1	0.3	0.2	0.4	0.5
Sphingobacteriia	0.0	0.0	0.0	0.0	0.2	0.3	0.2	0.2
Chlamydiae	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
Chloroflexi;KD4-96	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
Chloroplast	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5
Bacilli	83.6	16.2	91.1	4.4	68.2	13.1	75.5	13.4
Clostridia	3.2	7.0	1.0	0.5	1.5	1.6	2.1	1.2
Erysipelotrichia	0.2	0.6	0.0	0.0	0.0	0.0	0.1	0.1
Negativicutes	0.3	0.8	0.0	0.1	0.2	0.3	0.3	0.4
Gracilibacteria;Other	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
Planctomycetacia	0.1	0.3	0.3	0.2	0.8	0.9	1.4	1.7
Alphaproteobacteria	0.6	1.1	0.3	0.3	2.5	2.3	2.0	2.6

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

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

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

TM6 (Dependentiae); uncultured	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.2
bacterium								
Verrucomicrobiaceae	0.0	0.0	0.0	0.2	0.1	0.2	0.0	0.0
WS6;Other	0.0	0.0	0.0	0.1	0.1	0.6	0.0	0.0
Unassigned;Other	0.0	0.1	0.1	0.2	0.2	0.8	0.2	0.4
Genus								
Acidimicrobiales; OM1 clade;	0.0	0.0	0.0	0.0	0.2	0.4	0.4	0.8
uncultured bacterium								
Acidimicrobiales; uncultured; Other	0.0	0.0	0.0	0.0	0.0	0.1	0.4	1.3
Bifidobacterium	0.2	0.5	0.1	0.1	0.3	0.7	0.3	0.4
Corynebacterium 1	2.8	4.1	2.3	2.5	6.1	5.2	1.8	2.9
Mycobacterium	0.4	1.0	0.6	1.2	3.9	3.9	3.3	7.5
Nocardia	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Brevibacterium	0.8	0.9	0.4	0.5	0.0	0.1	0.1	0.2
Brachybacterium	0.1	0.2	0.1	0.2	0.0	0.0	0.0	0.1
Intrasporangiaceae;Other	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1
Leucobacter	0.3	0.4	0.1	0.2	0.2	0.4	0.1	0.2
Microbacteriaceae;Other	0.2	0.5	0.2	0.3	0.3	0.4	0.2	0.3
Arthrobacter	0.2	0.4	0.3	0.4	0.1	0.2	0.1	0.2
Glutamicibacter	0.2	0.6	0.1	0.2	0.0	0.1	0.0	0.1
Kocuria	0.3	0.4	0.2	0.2	0.1	0.3	0.2	0.2
Micrococcaceae;Other	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.1
Nocardioides	0.0	0.0	0.0	0.0	0.3	1.0	0.0	0.0
Propionibacterium	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.3
Collinsella	0.2	0.8	0.0	0.0	0.0	0.0	0.0	0.1
Enterorhabdus	0.0	0.1	0.0	0.0	0.3	0.8	0.0	0.0
Coriobacteriaceae; uncultured	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0
Solirubrobacterales; Elev-16S-1332	0.2	0.8	0.0	0.0	0.0	0.0	0.0	0.0
uncultured bacterium								
Bacteroides	0.1	0.4	0.0	0.1	0.0	0.1	0.2	0.4
Bacteroidales S24-7 group; uncultured	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
bacterium								
Prevotella 2	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0
Prevotella 9	0.7	2.2	0.0	0.1	0.0	0.0	0.1	0.2
Cloacibacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Flavobacterium	0.0	0.1	0.0	0.0	0.1	0.2	0.2	0.2
Flavobacteriaceae;Other	0.0	0.1	0.0	0.1	0.1	0.2	0.1	0.3
Sediminibacterium	0.0	0.0	0.0	0.0	0.1	0.2	0.1	0.2
Chlamydiales;Other	0.1	0.2	0.4	0.4	1.1	1.0	1.6	1.6
Chloroflexi; KD4-96; uncultured	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.2
bacterium								
Chloroplast;Other	1.5	2.8	0.2	0.5	5.4	4.0	1.9	2.5

Bacillus	0.5	0.4	0.5	04	0.8	0.6	0.5	04
Bacillaceae:Other	0.3	0.4	0.3	0.3	0.0	0.0	0.5	0.1
Brevibacillus	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1
Paenibacillus	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.1
Planococcaceae;Other	0.1	0.1	0.1	0.1	0.2	0.2	0.0	0.1
Staphylococcus	1.3	1.0	0.8	0.4	0.5	1.3	0.3	0.5
Staphylococcaceae;Other	0.1	0.2	0.1	0.2	0.1	0.2	0.0	0.1
Bacillales;Other	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
Granulicatella	0.0	0.1	0.0	0.1	0.1	0.2	0.1	0.2
Enterococcus	0.3	0.3	0.3	0.2	0.4	0.4	0.4	0.4
Lactobacillus	77.9	16.1	86.5	4.4	61.3	12.4	67.6	12.2
Leuconostoc	0.0	0.1	0.3	0.3	0.1	0.2	0.1	0.3
Weissella	0.4	0.8	0.7	0.9	0.4	1.3	2.8	2.8
Lactococcus	0.3	0.4	0.1	0.2	0.5	0.4	0.3	0.3
Streptococcus	1.6	1.6	1.2	1.4	3.6	3.5	2.9	2.2
Clostridium sensu stricto 1	0.4	0.7	0.1	0.1	0.0	0.1	0.1	0.1
Clostridiaceae 1;Other	0.2	0.4	0.1	0.2	0.3	0.4	0.2	0.3
Alkaliphilus	0.0	0.0	0.0	0.0	0.1	0.5	0.0	0.0
Clostridiales; Family XI; uncultured	0.1	0.2	0.0	0.1	0.0	0.2	0.0	0.1
Clostridiales; Family XI; Other	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.3
Blautia	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.2
Roseburia	0.2	0.6	0.0	0.2	0.0	0.0	0.0	0.0
Peptostreptococcaceae; Other	0.2	0.3	0.0	0.1	0.1	0.2	0.0	0.1
Faecalibacterium	0.3	1.0	0.0	0.1	0.0	0.0	0.1	0.3
Ruminococcaceae UCG-002	0.1	0.6	0.0	0.0	0.0	0.0	0.0	0.1
Ruminococcaceae UCG-005	0.2	0.7	0.0	0.0	0.0	0.0	0.0	0.0
Ruminococcus 2	0.1	0.4	0.0	0.1	0.0	0.0	0.0	0.1
[Eubacterium] coprostanoligenes group	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
Ruminococcaceae; uncultured	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.3
Clostridiales; Other	0.1	0.3	0.1	0.2	0.6	0.9	0.5	0.8
Phascolarctobacterium	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.1
Acidaminococcaceae;Other	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2
Megasphaera	0.0	0.1	0.0	0.1	0.2	0.2	0.1	0.1
Gracilibacteria; Othe	0.0	0.0	0.1	0.3	0.2	0.9	0.0	0.0
Planctomycetaceae; Pir4 lineage	0.0	0.0	0.1	0.1	0.2	0.2	0.2	0.0
Planctomyces	0.0	0.1	0.1	0.2	0.3	0.3	0.6	0.0
Planctomycetaceae; uncultured	0.1	0.3	0.1	0.2	0.2	0.4	0.4	0.1
Bradyrhizobium	0.0	0.0	0.0	0.0	0.7	1.6	0.1	0.0
Ochrobactrum	0.0	0.0	0.0	0.0	0.2	0.5	0.1	0.0
Hyphomicrobium	0.1	0.2	0.0	0.0	0.0	0.0	0.1	0.0
Mesorhizobium	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
Phyllobacteriaceae; Other	0.2	0.5	0.1	0.2	0.2	0.5	0.4	0.4

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