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

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

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Abstract

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

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

Keywords

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

1. Introduction

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

98 inclusion of ingredients such as FM has usually guaranteed a high acid-binding capacity
99 of diets. FM has one of the highest buffering capacities in comparison to most alternative
100 vegetable aquafeed ingredients such as sunflower meal, soybean meal, rapeseed meal and
101 glutens (Giger-Revendin et al., 2002) and its decreasing content to relatively low levels
102 in current formulations might affect the ideal gastrointestinal pH for digestive enzyme
103 action and gut bacterial community of specific fish species. Different levels and models
104 of regulation of gastrointestinal pH in fish species of commercial interest have been
105 described. Species such as rainbow trout (*Oncorhynchus mykiss*) and cobia
106 (*Rachycentron canadum***)**, are characterised by a continuous acid secretion and the
107 maintenance of a low gastric pH during fasting while others displayed neutral gastric pH
108 during fasting followed by hydrochloric acid released only after the ingestion of a meal
109 (Bucking and Wood, 2009; Yúfera et al., 2019). Ranges in pH of several fish species have
110 been reported between 2.0 and 7.0 for the stomach and between 6.2 and 8.2 for the
111 intestine mainly depending on fish specific feeding habits including omnivorous *vs*.
112 carnivorous and frequent feeders *vs*. less frequent feeders (Krogdahl et al., 2015; Yúfera
113 et al., 2012). In this contest to the best of our knowledge little information is available for
114 the European sea bass (*Dicentrarchus labrax*) which seems to display very low pH value
115 in the stomach followed by alkaline levels in the intestine (Nikolopoulou et al., 2011). In
116 this carnivorous species a reduction of feed BC induced by a very low FM dietary level
117 might led to relatively low pH in the intestinal tract with consequences on feed utilisation.
118 Several studies have shown that European sea bass can cope well with high levels of
119 FM protein replacement by a mixture of plant feedstuff alone or in combination with other
120 terrestrial animal protein sources using different level of fish oil (FO). Kaushik et al.
121 (2004), during a 12 week experiment, reported no adverse consequence in terms of growth

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

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

2. Materials and methods

2.1 Experimental diets

Three isonitrogenous (47% protein) and isolipidic (16% lipid) diets were formulated, two experimental diets with 10% FM with or without the inclusion of calcium carbonate ($CaCO_3$) (FM10+B, FM10, respectively) and one control diet containing 20% FM (FM20). $CaCO_3$ was added at 3% in FM10+B in order to achieve a higher buffering

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

2.2 Fish and rearing trial

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

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

2.3 Sampling

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. Specific growth rate (SGR), feed intake (FI) and feed efficiency (FE) were calculated. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 10 fish and on a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency rate (PER) and gross protein efficiency (GPE) were calculated. Furthermore, at the end of the trial, wet weight, viscera and liver were individually recorded for 5 fish per tank to determine viscerosomatic index (VSI) and hepatosomatic index (HSI). At the end of the trial, three fish per tank were sampled at 0, 4, 8 and 12 hours post meal (hpm) to measure gastrointestinal pH. The meal was supplied to satiation in keeping with the standard procedures employed during the trial. In addition at 4 and 8 hours post-prandial samples of the whole gastrointestinal tract (n=4/treatment) were stored at −80 °C and then freeze-dried for enzyme activity analysis. At the end of the trial

194 at 12 hpm samples of hindgut content from 3 fish per tank were collected individually for
195 gut bacterial community characterization. The gastrointestinal tract was dissected under
196 sterile conditions and the hindgut content was squeezed out into an Eppendorf tube (one
197 per fish) and placed at −80◦C until DNA extraction (Parma et al., 2016). Hindgut was
198 defined as the section from the ileorectal valve to the anus. Gut bacterial community
199 analyses were then performed on a pooled of 300 mg of intestinal content originating
200 from the 3 fish sampled for each tank (100mg of intestinal content from each individual
201 fish sampled) according to Parma et al. (2016).
202 All experimental procedures were evaluated and approved by the Ethical-Scientific
203 Committee for Animal Experimentation of the University of Bologna, in accordance with
204 European directive 2010/63/UE on the protection of animals used for scientific purposes.
205
206 *2.4 Calculations*
207
208 The formulae employed were as follows:
209 Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and
210 IBW represent the final and the initial body weights). Feed Intake (FI) (g kg ABW^{-1}
211 day^{-1}) = ((1000*total ingestion)/(ABW))/days)) (where average body weight, ABW =
212 (IBW + FBW)/2. Feed efficiency (FE) = wet weight gain/dry feed intake . Viscerosomatic
213 index (VSI) (%) = 100 * (viscera weight / body weight). Hepatosomatic index (HSI) (%)
214 = 100 * (liver weight / body weight). Protein efficiency rate (PER) = (FBW – IBW) /
215 protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW)
216 - (% initial body protein * IBW)] / total protein intake fish.
217

2.5 Gastrointestinal luminal pH measurements

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

2.6 Enzyme activity analysis

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

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

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

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

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

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

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

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

2.7 Gut bacterial community DNA extraction and sequencing

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

290 at 55°C for 30 s and extension at 72°C for 30 s, and a final extension step at 72°C for 5
291 min (Parma et al., 2016). PCR reaction were cleaned up for sequencing by using
292 Agencourt AMPure XP magnetic beads as recommended in the Illumina protocol "16S
293 Metagenomic Sequencing Library Preparation" for the MiSeq system, and as used in
294 several other publications (Biagi et al., 2018; Soverini et al., 2016). Raw sequences were
295 processed using the QIIME pipeline (Caporaso et al., 2010). After length
296 (minimum/maximum = 200/600 bp) and quality filtering with default parameters, reads
297 were binned into OTUs at a 0.97 similarity threshold using UCLUST (Edgar, 2010).
298 Assignment was carried out by using the RDP classifier against Greengenes database
299 (May 2013 version). Alpha-diversity analysis was performed using the observed OTUs,
300 Faith's PD, Chao1, Shannon index metrics and the species evenness calculated as the ratio
301 between the Shannon index and the natural logarithm of species richness (Observed
302 OTUs index). Beta-diversity was estimated by Bray-Curtis distances, which were used as
303 input for principal coordinates analysis (PCoA).
304
305 *2.8 Analytical methods*
306
307 Diets and whole body were analysed for proximate composition. Moisture content was
308 obtained by weight loss after drying samples in a stove at 105 °C until a constant weight
309 was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl
310 method and multiplying N by 6.25. Total lipids were determined according to Bligh and
311 Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant
312 weight in a muffle oven at 450 °C. Gross energy was determined by a calorimetric bomb
313 (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, U.S.A).

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

2.9 Statistical analysis

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

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

3. Results

3.1 Growth

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

3.2 Gastrointestinal luminal pH pattern

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

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

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

3.3 Digestive enzymes activity

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

3.4 Gut bacterial community profiles

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

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

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

4. Discussion

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

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

Low FM dietary level might reduce feed BC with possible effects on gastrointestinal luminal digestive conditions. In the present study, gastric pH was not significantly affected by 10% FM dietary level in comparison to a control diet. Our results confirm previous observations that European sea bass maintain a gastric pH slightly acidic (4.5-5) during fasting followed by a strong acidification (pH below 3) stimulated by the ingestion of food (Nikolopoulou et al., 2011). However, the authors reported the lowest gastric pH values (3-3.5) at 12 hpm while in the present study the levels dropped to 3 already at 4 hpm to reach the lowest values (2.5) at 8 hpm. Since the authors utilised a higher FM dietary level compared to the present study (40 to 60% FM dietary level) an expected higher feed BC might have determined those differences. The cause of the variation in gastrointestinal pH in the same species between experiments could also be related to fish size/age due to changes in feeding habit from juvenile to adults, which can affect digestion controls (Yúfera et al., 2004). However, no significant differences in gastrointestinal pH values have been reported from 7 up to 100 g sea bream juveniles and from 0.3 to 10 kg Atlantic salmon (*Salmo salar*) (Yúfera et al., 2004; Krogdahl et al., 2015). Focusing on the effects of the $CaCO_3$ inclusion at gastric level, the pH pattern of

458 FM10+B did not show a significant decreasing trend between 0 and 4 hpm (displayed in
459 the other treatments) indicating a possible feed BC effect able to slightly resist gastric
460 acidification during the first hours after a meal. Data on intestinal pH displayed neutral
461 to slightly acidic values indicating an effective active pancreatic exocrine HCO_3 secretion
462 in the anterior intestinal tract as previously reported by Nikolopoulou et al. (2011) for this
463 species fed one morning meal. However, these authors found an increasing tendency over
464 time with most intestinal pH values ranging between 7 and 8. Thus, the higher capability
465 of rapid alkalinisation at the intestinal level mentioned by them was less evident in the
466 present study. Probably, the two meals offered in the present study altered the feed transit
467 time and therefore the luminal pH, as it was observed in gilthead sea bream at different
468 moments or different daily feeding frequencies (Montoya et al., 2010; Yúfera et al.,
469 2014). While experimental diets did not affect pH values in the anterior and mid-intestine,
470 the addition of $CaCO_3$ in low FM diet led to a higher pH in the hindgut at 12 hpm
471 compared to FM10. This difference is also reinforced by the different temporal pH pattern
472 occurring in FM10 (no significant differences between 8 and 12 hpm) and FM10+B
473 (significant pH increasing between 8 and 12 hpm) for both mid-intestine and hindgut
474 indicating that the higher feed BC of FM10+B seems to facilitate the increase of pH
475 between 8 and 12 hpm in the mid-intestine and hindgut.

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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

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

	FM20	FM10	FM10+B
Ingredients, % of the diet			
Fish meal	20.0	10.0	10.0
Soybean meal	7.20	7.00	4.90
Soya concentrate	25.0	30.0	30.0
Wheat gluten	8.16	15.45	16.96
Corn gluten	7.00	2.01	3.58
Wheat	20.95	22.89	19.04
Fish oil	6.89	7.47	7.38
Rapeseed oil	4.59	4.98	4.92
Vit/Min premix[1]	0.20	0.20	0.20
$CaCO_3$	0.0	0.0	3.0
Lysine	-	0.11	0.11
Methionine	-	0.36	0.36
Proximate composition, % on a wet weight basis			
Moisture	5.83	5.76	5.67
Protein	47.1	46.6	46.6
Lipid	16.9	15.6	16.1
Ash	5.77	5.15	7.30
Gross energy, cal g^{-1}	4975.7	4985.4	4923.1
pH	6.4	6.4	6.5
Buffering capacity[2]	0.75	0.53	0.75

[1] Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011)
[2] Amount (ML) 1 N HCL to lower 1 g of the diet to pH 3
$CaCO_3$ = calcium carbonate
FM = fishmeal
B = buffering additive

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

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

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffer (B, $CaCO_3$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 * (ln FBW- ln IBW) / days.
ABW = average body weight = (IBW + FBW)/2.
FI= Feed intake (g kg ABW^{-1} day^{-1}) = ((1000*total ingestion)/(ABW))/days)).
FE = Feed efficiency = wet weight gain/dry feed intake

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

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

Data are given as the mean (n=3; n=15 for VSI, HSI) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffer (B, $CaCO_3$)
VSI = Viscerosomatic index (%) = 100*(viscera weight/final body weight, FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
PER = Protein efficiency ratio = ((FBW-initial body weight, IBW)/protein intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.

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

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

Data are given as the mean (n=4) ± SD. Enzyme activity of the different experimental groups after 64 days of feeding, expressed in U g^{-1} body weight. Pepsin, amylase and lipase = U g^{-1} BW $*10^{3}$.
Different upper-case letters indicate significant difference (Student's T-test, $P \leq 0.05$) within the same diet at different sampling time.
FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffer (B, $CaCO_3$)
Time 4 = 4 hours post meal, hpm; Time 8 = 8 hpm

Table 5. Alpha-diversity metrics values of gut bacterial community of European sea bass juveniles fed different diets. Reported data are expressed as means ± SD (n = 3)

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

Data are given as mean ± SD (n = 3)
OTU = operational taxonomic unit
FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffer (B, $CaCO_3$).
No significant difference among treatment were detected (Mann-Whitney-Wilcoxon test, $P \leq 0.05$)

Figure captions

Figure 1.

Gastrointestinal pH (mean, n=9 ± standard deviation, SD) measured in the stomach (A), anterior intestine (B), mid-intestine (C) and hindgut (D) during 12 hours post prandial cycle in European sea bass juveniles fed different diets. (FM20 ———) = 200g kg^{-1} fishmeal (FM); (FM10 - - -) = 100g kg^{-1} FM; (FM10+B ·····) = 100g kg^{-1} FM + 30g kg^{-1} buffering additive (B, CaCO3). Different lower-case letters (a b; a' b'; a''b''; FM20, FM10, FM10+B, respectively) indicate significant difference between sampling time while different upper-case letters indicate significant difference between treatments at each sample time (Two-way ANOVA, $P \leq 0.05$) .

Figure 2

Relative abundance (%) of gut bacteria composition at phylum (A) and family levels (B) measured in each tank in European sea bass juveniles fed different diets after 64 days. In each tank analyses were performed on one pool of hindgut content originating from 3 fish sampled per tank. FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffering additive (B, CaCO3). Only taxa with relative abundance > 0.1% in at least 1 sample were included.

Figure 3

Principal Coordinates Analysis (PCoA) plot based on the Bray-Curtis distances representing the spatial distribution of the gut bacterial community belonging to the three dietary treatments (FM20 – green; FM10+B - cyan and FM10 - red). FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffering additive (B, CaCO3). FM20 was significantly separated both from diet FM10+B (p value

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

Figure 4

Relative abundance (> 1%) of the most represented gut bacteria genera belonging to Lactic acid bacteria (LAB) found in European sea bass juveniles fed different diets. FM20 = 200g kg^{-1} fishmeal (FM); FM10 = 100g kg^{-1} FM; FM10+B = 100g kg^{-1} FM + 30g kg^{-1} buffering additive (B, CaCO3).

Figure 1

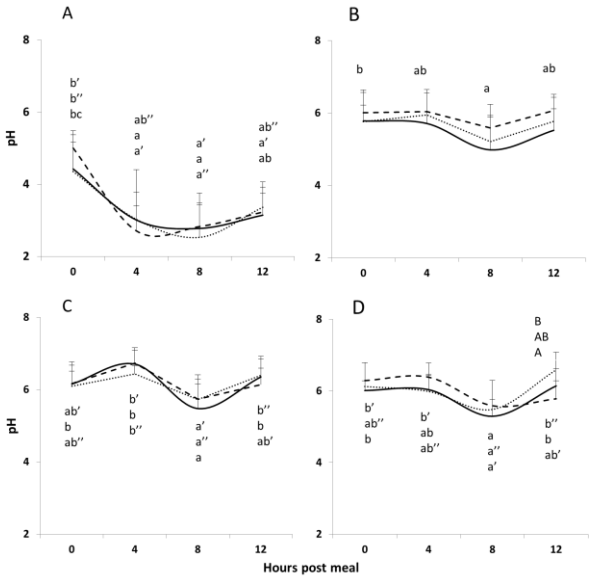
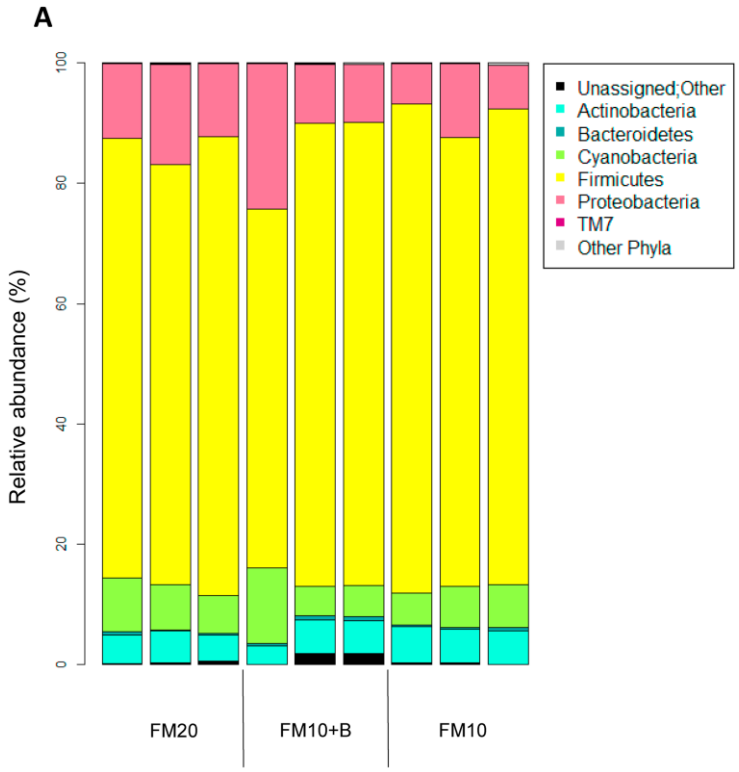

A
B
C
D
pH
Hours post meal
0
4
8
12
2
4
6
8
b′ b″ bc
ab″ a a′
a′ a a″
ab″ a′ ab
b ab a ab
ab′ b ab″
b′ b b″
a′ a″ a
b″ b ab′
b′ ab″ b
b′ ab ab″
a a″ a′
B AB A
b″ b ab′

Figure 2A


A
Relative abundance (%)
0
20
40
60
80
100
FM20
FM10+B
FM10
Unassigned;Other
Actinobacteria
Bacteroidetes
Cyanobacteria
Firmicutes
Proteobacteria
TM7
Other Phyla

941 Figure 2B
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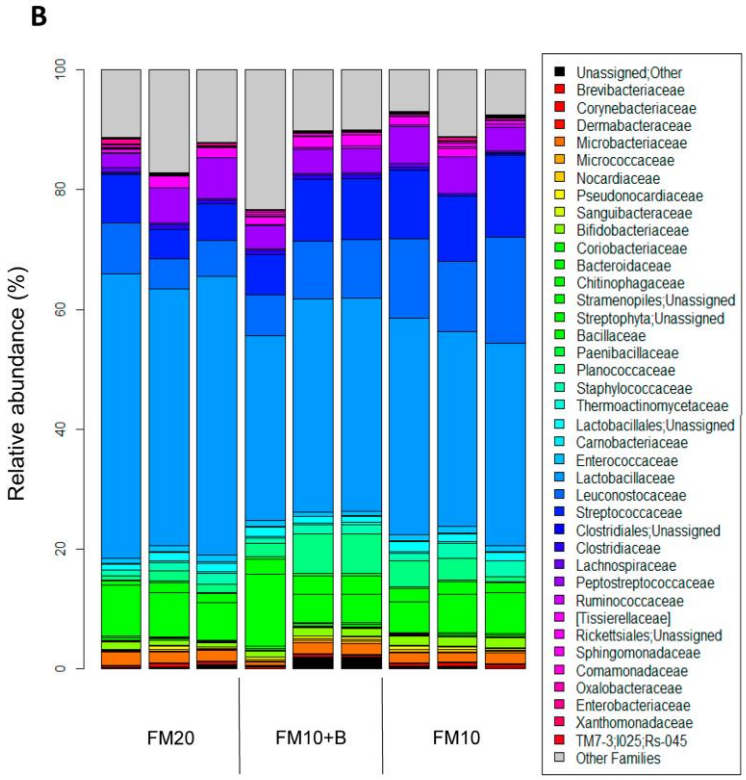

944
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946 Figure 3
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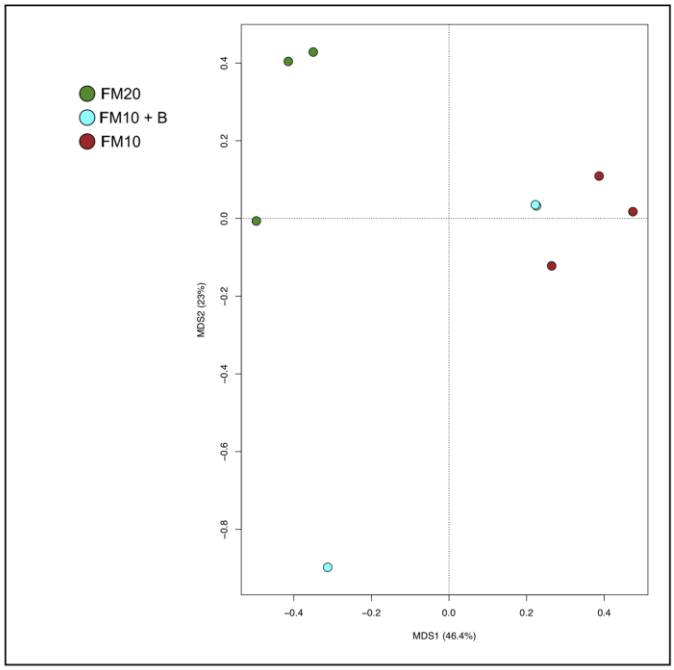

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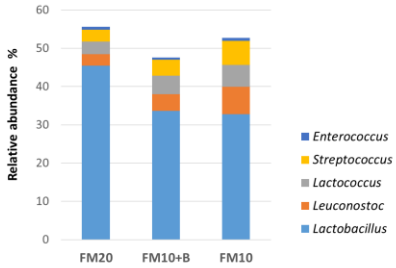
950 Figure 4



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