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Next-generation sequencing characterization of the gut bacterial community of gilthead sea bream (*Sparus aurata*, L.) fed low fishmeal based diets with increasing soybean meal levels

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26 histology.

27

Abstract

The present study was carried out to evaluate growth, gut histology and gut bacterial community of gilthead sea bream (*Sparus aurata*) fed with increasing dietary soybean meal (SBM) levels in a low fishmeal (FM) based diet, in comparison with a control diet. Five isoproteic and isolipidic experimental diets were formulated to contain increasing levels of SBM (0, 100, 200, and 300 g kg^{-1} named S0, S10, S20 and S30, respectively) with 150 g kg^{-1} of FM, and one control diet (C) without SBM and containing 350 g kg^{-1} of FM. Sixty sea bream (initial body weight 75.9 ± 1.9 g, n = 900) per tank were reared in a recirculation system at 23.0 ± 1.0 °C and fed to satiation. The trial was run in triplicate and lasted 100 days. At the end of the trial fish fed the S30 diet showed a higher ($P \leq 0.05$) specific growth rate (SGR) compared to S0 (SGR, 1.17 ± 0.03, 1.20 ± 0.01, 1.22 ± 0.01, 1.25 ± 0.01 and 1.21 ± 0.04 for S0, S10, S20, S30 and C, respectively), and a higher feed intake (FI) compared to S0, S10 and S20. Sea bream fed the C diet had a higher ($P \leq 0.05$) FI compared to S0 (FI, 1.40 ± 0.01, 1.45 ± 0.01, 1.44 ± 0.03, 1.51 ± 0.03 and 1.46 ± 0.02 for S0, S10, S20, S30 and C, respectively). No significant differences in feed conversion rate, protein efficiency ratio, gross protein efficiency and gross lipid efficiency among the treatments were detected. No specific histopathological changes indicative of soy-induced enteritis were observed in the intestine of any fish

examined. Gut bacterial community of the distal intestine content was analyzed by Next-Generation Sequencing. At the phylum level, the gut bacterial community was dominated by *Firmicutes* (relative abundance 71%), while the most represented family was *Lactobacillaceae* (26%). Even if no significant differences ($P \leq 0.05$) in the gut bacterial community α and β-diversity according to the different diets were detected, *Cyanobacteria* and *Lactobacillaceae* progressively increased from diet C to diet S30. In conclusion results of growth, nutrient utilization, gut histology and gut bacterial community indicate that SBM can be successfully incorporated up to a level of 300 g kg^{-1} with the inclusion of 150 g kg^{-1} of FM, without any deleterious effects on growth, protein utilization and gut health during the on-growing of sea bream.

77 **1. Introduction**

78

79 Gilthead sea bream is one of the most important species for
80 European aquaculture, representing around 51% of the total
81 finfish marine production in the Mediterranean area (FAO,
82 2010). Due to the current economic downturn and the
83 fluctuation of the gilthead sea bream market, a reduction in
84 feed costs while ensuring optimal growth and fish health is
85 essential to maintain the profitability of its farming (Martinez-
86 Llorens et al., 2009; Mongile et al., 2014). In this context, the
87 importance of vegetable protein is well recognized by feed
88 industry operators due to the growing pressure for alternative
89 fishmeal (FM) substitutes in fish diets. Among the different
90 ingredients, soybean meal (SBM) is one of the most interesting
91 alternative FM because of the advantages of supply, price, and
92 protein and amino acid composition (Bonaldo et al., 2008).
93 However this ingredient may induce a variety of histological
94 and functional changes in the gastrointestinal tracts of fish,
95 especially in salmonids, including morphological alterations
96 and inflammation (Krogdahl et al., 2003, 2010). These changes
97 may be due to direct effects of anti-nutritional factors in plant
98 ingredients and/or the indirect result of diet-induced changes in
99 the structure and function of the intestinal bacterial community
100 (Olsen et al., 2001; Ringø et al., 2006).

Previous studies on gilthead sea bream have shown that the optimum dietary SBM levels, using a dietary FM content higher than 200 g kg^{-1}, were 205 g kg^{-1} for maximum growth (Martinez-Llorens et al., 2009). Further increasing the level of SBM up to 300 g kg^{-1} of the diet had no significant effects on the specific growth rate (SGR), feed intake (FI) and feed conversion rate (FCR) in juvenile specimens of the same species, although high SBM level led to some changes in the distal intestine, with the presence of cellular infiltration of the submucosa and lamina propria (Bonaldo et al., 2008).

In this context the exploration of fish gut bacterial community can represent an emerging tool to evaluate the application of vegetal ingredients in fish feed formulations. Increased knowledge of the human gut microbiota is driving research into development, immunity, disease, lifestyle and nutrition (Furusawa et al., 2013). Similarly, the knowledge and manipulation of the gut microbiome in teleosts, especially in aquaculture, could be potentially addressed through nutrient digestion, synthesis, absorption, pathogen resistance, growth, sexual maturation, morphogenesis and survivorship (Llewellyn et al., 2014). To date, our understanding of the teleost gut bacterial community and of its functional significance has lagged well behind that of humans and other terrestrial vertebrates (Ray et al., 2012). Most understanding of the intestinal microbiota of fish is largely derived from culture-

126 based approaches and 16S rRNA gene fingerprinting methods
127 such as denaturing gradient gel electrophoresis (DGGE).
128 However, these methods usually reveal only a limited range of
129 microbial diversity (Desai et al., 2012; Carda-Diéguez et al.,
130 2014). Next-Generation Sequencing (NGS) has been used in
131 recent years to examine the gut microbiome of humans,
132 terrestrial and marine vertebrate including some fish species as
133 recently reviewed by Ghanbari et al. (2015). However, only for
134 a few species such as rainbow trout *Oncorhynchus mykiss*,
135 Siberian sturgeon *Acipenser baerii* and zebrafish *Danio rerio*,
136 was this technique applied to explore the impact of diet on the
137 gut bacterial community (Desai et al., 2012; Semova et al.,
138 2012; Geraylou et al., 2013). In sea bream, *Sparus aurata*, data
139 on gut bacterial community using NGS have been recently
140 published regarding fish fed exclusively fishmeal or vegetable
141 protein based diets (Estruch et al., 2015), while no data are
142 available for this species fed increasing SBM levels in practical
143 diet formulations.

144 Furthermore few studies have explored in this species the
145 effects of increasing levels of SBM on performance using low
146 FM based diets as the only animal protein source and most of
147 the data on literature were restricted to replace FM with SBM.
148 At this regards, we evaluated the effects of SBM by replacing a
149 mixture of vegetal ingredients, wheat meal (WM), wheat gluten
150 (WG), corn gluten (CG) and sunflower meal (SM) which are

currently used in practical formulation at industrial level to determine the optimal inclusion rate in practical low fish meal diet.

The aims of this study were: 1) to evaluate the effects of dietary inclusion of SBM and a low FM content in practical diet formulations on growth, nutrient utilization and gut histology of gilthead sea bream; 2) to evaluate changes in the gut bacterial community of gilthead sea bream fed practical diets with increasing levels of SBM and a low FM content, in comparison to a control diet.

2. Materials and methods

2.1. Diets

Ingredients and proximate composition of the experimental diets are presented in Table 1. Four isoproteic and isolipidic diets were formulated with practical ingredients to contain increasing levels of SBM (0, 100, 200, and 300 g kg^{-1}, named S0, S10, S20, and S30, respectively) with a low FM content (150 g kg^{-1}), while a control diet (C) was formulated to contain 0 g kg^{-1} SBM and 350 g kg^{-1} FM content. SBM was replaced by adding WM, WG, CG and SM. The diets were manufactured by Skretting Aquaculture Research Centre (Stavanger, Norway) using extrusion technology. According to

the feed manufacturer, the protein and lipid levels were within the range of the commercial diets for sea bream as well as the FM level in the C group which was chosen as optimal standard level for commercial diet of this species. All feeds were produced as extruded sinking pellets (specific gravity 1.15) with a diameter of 4 mm.

2.2. Fish, experimental set-up and sampling

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Sea bream with an initial average weight of 75.9 ± 1.9 g were obtained from the hatchery Panittica Italia, Fasano, Italy. Before the experiment, fish were acclimated for 2 weeks to the experimental tanks and fed a mix of the experimental diets. At the beginning of the trial, 60 fish per tank were randomly distributed into 15, 1000 L square conical bottom tanks to obtain five triplicate fish groups, each per dietary treatment. Tanks were provided with natural seawater and connected to a closed recirculation system consisting of a mechanical sand filter (Astralpool, Spain), an ultraviolet light (Philips, the Netherlands) and a biofilter (Astralpool, Spain). The water exchange rate within each tank was 100% every hour. The water renewal of the total system was 5 % daily. Mean water temperature was maintained at 23.0

± 1.0 °C throughout the experiment; photoperiod was held constant at a 12 h day length through artificial light (300 lux at the water surface — Delta Ohm luxmeter HD-9221; Delta-Ohm, Padua, Italy). The oxygen level was kept constant (8.0 ± 1.0 mg L^{-1}) by a liquid oxygen system connected to a software controller (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen, TAN ≤ 0.1 mg L^{-1}), nitrite (NO_2 ≤ 0.2 mg L^{-1}) and nitrate (NO_3 ≤ 50 mg L^{-1}) were determined spectrophotometrically once a day (Spectroquant Nova 60, Merk, Lab business) at 12.00 p.m. At the same time, pH (7.8–8.2) and salinity (28-33 g L^{-1}) were determined. The feeding trial lasted a total of 100 days. Fish were overfed by automatic feeders twice a day with a 5-10 % overfeeding ration for six days a week, while one meal was supplied on Sundays. Each meal lasted 1 hour and after that the uneaten feed was trapped by a feed collector at the water output of tanks, dried overnight at 105°C and the weight deducted from the feed intake for overall calculations.

At the beginning and at the end of the experiment, all the fish of each tank were individually weighed. At the end of the trial digesta samples from 3 fish per tank were collected individually. The gastrointestinal tract was dissected under sterile conditions and the distal gut content was squeezed out into an Eppendorf tube (one per fish) and placed at −80 °C until DNA extraction (Desai et al., 2012).

Carcass proximate composition was determined on a pooled sample of ten fish collected at the beginning of the trial and on pooled samples of five fish per tank collected at the end of the trial. Furthermore, at the end of the trial, wet weight of viscera and liver was individually recorded from five fish per tank to determine visceral (VSI) and hepatosomatic (HSI) indices.

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

2.3. Gut histology

At the end of the trial 15 animals per treatment were randomly sampled. After euthanasia with a lethal dose of 2-phenoxyethanol, the gut was removed and the intestine was divided into two segments, proximal and distal; from each segment a 5 mm-long piece was sectioned and fixed in 10% buffered formalin. Samples were then processed for routine histology to obtain 3 µm thick transverse sections, which were stained with haematoxylin-eosin (H&E). Sections were evaluated under a light microscope (Nikon Eclipse 80i).

2.4. Gut bacterial community 16S sequencing

Total bacterial DNA was extracted from a pool of distal intestine content obtained from 3 fish per tank (100 mg of distal intestine content per fish) as reported by Schnorr et al. (2014). PCR amplifications of the V3-V4 region of the 16S rRNA gene were carried out in 25 µl volumes with 25 ng of microbial DNA, 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems), and 200 nM of the primers S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (Klindworth et al., 2013) including Illumina overhang adapters. Reaction conditions were as follows: initial denaturation at 98°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with a final extension step at 72°C for 5 min. Amplicons were purified using Agencourt AMPure XP magnetic beads. This magnetic bead-based system is recommended in the Illumina protocol "16S Metagenomic Sequencing Library Preparation" for the MiSeq system, and has been used in several other publications (Soverini et al., 2016). According to the Illumina protocol, 20% PhiX control was used. Indexed libraries were prepared by using Nextera technology and cleaned up with Agencourt® magnetic beads. The final libraries were pooled at equimolar concentrations, denatured and diluted to 6 pM before loading onto the MiSeq flow cell. Sequencing was performed on Illumina MiSeq platform using a 2 × 300 bp paired end protocol, according to

the manufacturer's instructions (Illumina, San Diego, CA). Raw sequences were processed using the QIIME pipeline (Caporaso et al., 2010). After length (minimum/maximum = 300/600 bp) and quality filtering with default parameters, reads were binned into OTUs at a 0.97 similarity threshold using UCLUST (Edgar, 2010). Assignment was carried out by using the RDP classifier against Greengenes database (May 2013 version). Alpha-diversity rarefaction curves were performed using the Faith's phylogenetic diversity, Chao1, observed species, and Shannon index metrics. Beta-diversity was estimated by weighted and unweighted UniFrac distances, which were used as input for principal coordinates analysis (PCoA).

2.5. Analytical methods

Diets and whole body samples were analyzed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 °C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method and multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant weight in a muffle oven at 450 °C.

2.6. Calculations

The formulae employed were as follows:

Specific growth rate (SGR) (day^{-1}) = 100 * (ln FBW−ln IBW)/days (where FBW and IBW represent the final and the initial body weights).

Feed intake (FI) (% day^{-1}) = 100 * (crude feed intake/ ABW/day) (where ABW (g) = average body weight = (FBW + IBW)/2).

Feed conversion ratio (FCR) = feed intake/weight gain.

Visceral somatic index (VSI) (%) = 100 * (viscera weight/body weight).

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

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

Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) − (% initial body protein *IBW)]/total protein intake $fish^{-1}$.

Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) − (% initial body lipid * IBW)]/total lipid intake $fish^{-1}$.

2.7. Statistics

Data of growth performance, VSI, HSI, and nutritional indices are presented as mean ± standard deviation (SD) of three replicate groups and were analyzed by a one-way ANOVA followed by a Tukey's multiple comparison test. Statistical analysis of gut bacterial community was carried out by using R packages Stats and Vegan. Significant differences in the relative abundance of gut bacterial community components were obtained by Kruskall-wallis test. Data separation in the PCoA was tested using a permutation test with pseudo F-ratios (function Adonis in the Vegan package).

3. Results

3.1. Growth and histology

Growth performance is summarized in Table 2. At the end of the trial fish fed the S30 diet showed a higher ($P \leq 0.05$) SGR compared to S0 and a higher FI compared to S0, S10 and S20. Sea bream fed the C diet had a higher ($P \leq 0.05$) FI compared to S0, while no significant differences in FCR among the treatments were detected (Table 2). No significant differences in VSI, HSI, whole body composition and the nutritional indices PER, GPE, GLE, were observed among the treatments (Table 3). No specific histopathological changes

indicative of soy-induced enteritis were observed in the intestine of any fish examined (Fig. 1).

3.2. Gut bacterial community characterization

Fifteen pools of distal intestine content were analyzed by NGS of the V3 and V4 regions of the 16S rDNA gene. A total of 5,584,914 high quality reads were obtained from the starting 15,956,896 reads obtained, ranging from a minimum of 93,673 to a maximum of 687,596 reads per sample, with an average of 372,327 reads per sample. Further information about the number of reads for each sample and the coverage are reported in Supplementary Table 1. The number of reads across samples was normalized basing on the sample with the lowest number of reads and singletons were omitted from the analysis. Reads were clustered into 13,099 operational taxonomic units (OTUs) at 97% of identity, of which a total of 5,525 diet-specific OTUs were found (1,082 for diet S30; 1,016 for diet S20; 1,038 for diet S10; 833 for diet S0; 1,556 for control diet). Different metrics have been utilized to calculate α-diversity, including phylogenetic diversity, OTU species count, Chao 1 index for microbial richness and Shannon index for biodiversity (Fig. 2a). Rarefaction curves of the phylogenetic diversity approximated saturation, indicating a good coverage of the gut bacterial community. No differences in the gut bacterial

community α-diversity according to the different diets were detected (Fig. 2b).

At the phylum level, the average sea bream gut bacterial community is dominated by *Firmicutes* (relative abundance (rel. ab.) 71%), *Actinobacteria* (rel. ab. 9%), *Bacteroidetes* (rel. ab. 7%) and *Proteobacteria* (rel. ab. 6%), while *Cyanobacteria* (rel. ab. 3%) and *Verrucomicrobia* (rel. ab. 3%) were subdominant (Fig. 3a). The most represented families are: *Lactobacillaceae* (rel. ab. 26%), *Ruminococcaceae* (rel. ab. 12%), *Lachnospiraceae* (rel. ab. 10%) and *Clostridiales* families (rel. ab. 7%) (Fig. 3b). Among the subdominant families the most represented were, *Streptococcaceae* (rel. ab. 3%), *Cyanobacteria* (rel. ab. 3%), *Staphylococcaceae* (rel. ab. 3%), *Verrucomicrobia* (rel. ab. 3%) and *Enterobacteriaceae* (rel. ab. 2 %).

In order to highlight the impact of the different diets (S0, S10, S20, S30 and C) on the gut bacterial ecology of sea bream, we performed the PCoA analysis of the UniFrac distances among the gut bacterial community profiles (Fig. 4). Even though no significant differences among dietary groups were detected, both weighted and unweighted PCoA showed a tendency toward a samples separation according to the different diets. Fig. 5 shows the relative abundance of bacteria composition per sample at phylum (a) and family (b) levels, while in Fig. 6 we report the gut bacterial community components which showed

a different abundance in the different dietary groups. In particular, the abundance of *Cyanobacteria* progressively increased from diet C to diet S30 (Fig. 6a), while *Synergistetes* tend to show an opposite trend (Fig. 6b). Differently, *Actinobacteria* showed a higher abundance in diets S0 and S30 (Fig. 6c). Although there were no statistically significant effects, the *Lactobacillaceae* family was highly represented in fish fed S30 (Rel. ab. 43.3%) compared to those fed C diet (Rel. ab. 11.2%) (Fig. 6d).

4. Discussion

The inclusion of SBM at 100, 200 and 300 g kg^{-1} (S10-S30) of the diet with a low FM content (150 g kg^{-1}) led to equal growth and protein utilization in comparison to a control diet without SBM and having 350 g kg^{-1} of FM. The present results are in agreement with previous studies which have demonstrated the feasibility of including up to 300, 390 and 395 g kg^{-1} SBM in diets for on-growing sea bream without negative effects on growth and nutritive efficiency (Bonaldo et al., 2008; Martinez-Llorens et al., 2009; Kokou et al., 2012), although FM levels in these studies were higher than in the present trial or amino acid supplements were used. In the present study the lack of differences in the SGR, FCR, PER and GPE between S10, S20, S30, the C diet suggests that the

425 inclusion of 150 g kg^{-1} of FM in combination with SBM, WG
426 and CG will supply sufficient protein quality for this species.
427 Similarly, Dias. et al. (2009), showed that the growth
428 performance of sea bream towards the end of the grow-out
429 phase can be sustained by a practical dietary formulation
430 containing plant protein-derived and as little as 13% of marine-
431 derived proteins. However, in that study AA supplementation
432 and haemoglobin powder were also incorporated in the feed
433 while in the present study FM was the only animal protein
434 source. Focusing on the diets at low FM level (S0, S10, S20,
435 S30), fish fed S30 showed a higher SGR compared to those fed
436 S0. This seems mainly due to an increment of FI with
437 increasing dietary content of SBM. The reduced FI commonly
438 observed in fish given feeds containing plant protein may be
439 related to a reduced feed palatability and, in this regard, the use
440 of several mixtures of plant protein should reduce the potential
441 inhibition of feed consumption due to the specific effect of a
442 single ingredient (Fournier et al., 2004). Other studies reported
443 an increased feed consumption with increasing dietary levels of
444 SBM assuming that fish to meet their energy needs would have
445 increased the FI for a reduced available energy content as SBM
446 inclusion increased (Venou et al., 2006; Kokou et al., 2012).
447 SBM contains about 20% of non-starch polysaccharides (NSP)
448 and 10% oligosaccharides (Snyder and Kwon, 1987; Bach
449 Knudsen, 1997), which are considered indigestible by fish

compared to wheat and glutens. Therefore, despite the isoenergetic content of the diets, a reduction of available energy content would be expected at higher SBM inclusion level (Kokou et al., 2012). However, possible action of the gut bacterial community could allow part of SBM energy originating from NSP to be available to the fish in the form of low molecular weight fatty acids (Kihara and Sakata, 2002; Mountfort et al., 2002; Refstie et al., 2005; Kokou et al., 2012).

Gut histology revealed no specific histopathological changes indicative of soy-induced enteritis in the intestines of any fish examined. In a previous study on sea bream the inclusion of 30% SBM seemed to cause moderate and diffused expansion of lamina propria in the distal intestine due to an increase of mononuclear cell infiltration when compared to other treatments with 18 and 0% of SBM (Bonaldo et al., 2008). A dilatation of the submucosa by eosinophilics cells infiltration was also found in the distal intestine of sea bream fed diet containing bioprocessed SBM at the 40 and 60% levels (Kokou et al., 2012). However both studies were conducted at juveniles stage (weight range, 17.4 - 96.0 g and 15.7 - 48.9 g, respectively) compared to the on-growing stage of the present study (weight range 75.1 - 259.5 g). The inclusion levels of SBM seem to be better tolerated by fish at on-growing phase as supported by Martinez-Llorens et al. (2007) which concluded that dietary SBM might be included in the diets up to 30% in

juveniles and up to 50% in grow-out fish without affecting animal performance. In addition sea bream in grow-out phase showed high tolerance for soy saponins while in juvenile sea bream fed diets containing phytosterols and soy saponins some disturbances of the intestinal mucosa were observed (Couto et al., 2014 a, b); however, the histomorphological changes observed were very mild and, although statistically significant, the differences were judged to be minor and to represent normal adaptation to changes in diet composition (Couto et al., 2014a).

In the present study the gut bacterial community was characterized. According to our findings, the gut bacterial community is widely dominated by *Firmicutes* (rel. ab. 71%), showing *Actinobacteria* as the second dominant phyla (rel. ab. 9%). *Bacteroidetes, Proteobacteria* and *Cyanobacteria* were subdominant components with a relative abundance ranging from 3 to 7 % of the bacterial community. Our data are in general agreement with the previous Next Generation Sequencing-based survey of the gut bacterial community in sea bream (Estruch et al., 2015). Further, by mean of pyrosequencing of the V1-V3 region of the 16S rDNA, the Authors showed a co-dominace of *Actinobacteria* (rel. ab. 35%), *Proteobacteria* (rel. ab. 32%) and *Firmicutes* (rel. ab. 24%) in the hindgut bacterial community. The dominance of *Firmicutes* we observed in the sea bream analyzed in the

500 present study may be imputed to their specific dietary regimen
501 and rearing conditions, which represent environmental
502 variables known to mold the compositional structure of the gut
503 bacterial community. According to our findings, the gut
504 bacterial community of sea bream was enriched in several
505 fibrolytic *Firmicutes*, such as *Ruminococcaceae*,
506 *Lachnospiraceae* and *Clostridiales*. By producing butyrate
507 from indigestible complex polysaccharides, these
508 microorganisms may provide important beneficial functions for
509 the host (Nicholson et al., 2012). Indeed, butyrate plays
510 multiple roles in host physiology, being strategic for the
511 amelioration of energy extraction from diet, for the
512 reinforcement of the gut epithelium barrier as well as for
513 modulation of the host immune function (Petersson et al., 2011;
514 Arpaia et al., 2013; Russell et al., 2013). In addition, our
515 finding of *Cyanobacteria* in the sea bream gut bacterial
516 community is of particular interest in the context of the recent
517 findings by Di Rienzi et al. (2013). The Authors performed the
518 first whole genome reconstruction of *Cyanobacteria* detected in
519 the gut and proposed their specific designation as a new
520 candidate sibling phylum named *Melainabacteria*. Differently
521 from environmental *Cyanobacteria*, gut *Melainabacteria* are
522 non-photosynthetic and non-respiratory, while, according to the
523 authors, these microorganisms are obligate anaerobic
524 fermenters capable to relay on the different carbon sources

present in the gut. Analogous to certain *Firmicutes*, *Melainabacteria* can ferment plant polysaccharides in the gut, and being able to provide the host with B and K vitamins, these microorganisms have been included among the mutualistic components of the gut bacterial community (Di Rienzi et al., 2013).

Our finding showed only a subtle impact for the different diets on the overall gut bacterial composition of sea bream, as shown by PCoA analysis. However, evidence suggesting the impact of different levels of SBM on specific components of the gut bacterial community was obtained. At phylum level, increasing SBM dietary levels seem to favor the increase of *Cyanobacteria* and a correspondent decrease in *Synergistetes*. While the first is considered as a mutualistic gut bacterial community component able to provide the host with essential vitamins, *Synergistetes* act as opportunistic pathogens in the gut (Marchandin et al., 2010). Moreover, within the phylum of *Firmicutes* the fish fed a high level of SBM (S30) were enriched with the family of *Lactobacillaceae*, compared to those fed the control diet. The functional impact of lactic acid bacteria on fish intestine is still unclear, but potentially they may have beneficial effects on the immune system, could protect the fish against pathogenic invasion through the intestinal surface, are probiotic candidates and are generally considered as organisms associated with a healthy intestinal

epithelium (Cai et al., 1998; Nayak, 2010; Salinas et al., 2008; Dimitroglou et al., 2009; Ingerslev et al., 2014). Interestingly, in rainbow trout, Wong et al. (2013) described a trend of taxa within the phylum *Firmicutes* that were significantly discriminatory for diet type in which the relative abundance of *Lactobacillaceae* was enriched in fish fed a grain-based diet. Also the cichlid, *Astatotilapia burtoni*, which mostly feeds on plants and algae, exhibited most of the gut microbial biodiversity seen in cichlids with several nearly exclusive bacterial taxa such as *Lactobacillales* and gut *Melainabacteria* (Baldo et al., 2015).

What favors the presence of *Lactobacillaceae* in fish fed a plant diet is not well known, but some studies have shown that polyunsaturated fatty acids depress the intestinal lactobacilli population in fish (Ringø, 1993) in accordance with the more recent finding of Ingerslev et al. (2014), where a significantly lower amount of lactic acid bacteria was found in rainbow trout fed a marine-based diet compared to the fish fed a plant-based diet containing rape seed oil and pea meal. In contrast, in sea bream total fishmeal replacement with plant protein had a negative effect on the relative abundance of *Firmicutes* throughout the gut, particularly on the lactic acid bacteria *Lactobacillus* and *Streptococcus* (Estruch et al., 2015). *Lactobacillus* species are well equipped to metabolize oligosaccharides that occur in their habitats, such as sucrose,

575 stachyose and raffinose which are contained in soybeans at
576 approximately 10 % (Espinosa-Martosy and Rupérez, 2006;
577 NRC, 2011; Gänzle and Follador, 2012). Moreover,
578 *Lactobacillus* can benefit from simple sugars derived from
579 primary degraders in the gut, establishing syntrophic networks.
580 Thus, in the context of our research, it is reasonable to
581 hypothesize that the *Lactobacillaceae* growth could be
582 supported by these oligosaccharides.
583

584 **5. Conclusion**

585

586 In conclusion results of growth, nutrient utilization and gut
587 histology indicate that SBM can be successfully incorporated
588 up to a level of 300 g kg^{-1} with the inclusion of 150 g kg^{-1} of
589 FM as the only animal protein source, without any deleterious
590 effects on growth, protein utilization and gut health during the
591 on-growing phase.

592 A deep sequencing of the gut bacterial community of sea
593 bream during the on-growing phase was successfully obtained.
594 For the first time in this species, the gut bacterial community
595 was analyzed by NGS in fish fed increasing SBM levels using
596 practical current formulations. The overall gut bacterial
597 community was largely dominated by *Firmicutes*, including
598 several fibrolytic bacteria, supporting the hypothesis that this
599 species could be predisposed to digest plant-based ingredients.

A minimal impact of increasing dietary SBM levels on the overall gut bacterial community was observed. However SBM seems to favor positively specific components of the gut bacterial community such as *Cyanobacteria* and *Lactobacillaceae* which may provide important beneficial functions for the host and be associated with a healthy intestinal epithelium.

Conflicts of interest

The authors declare no conflicts of interest.

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Figure captions

Figure 1: histology of sea bream foregut (a,c,e,g,i) and hindgut (b,d,f,h,l). Control diet, C (a,b); 0 g kg^{-1} SBM diet, S0 (c,d); 100 g kg^{-1} SBM diet, S10 (e,f); 200 g kg^{-1} SBM diet, S20 (g,h) and 300 g kg^{-1} SBM diet, S30 (I,l). Intestine does not show any differences in terms of inflammatory or degenerative changes among diets (H&E, 20x objective).

Figure 2 a, b: OTUs rarefaction curves carried out with different α-diversity metrics (Faith's phylogenetic diversity (PD whole tree), observed OTUs, the Chao1 measure of microbial richness, and the Shannon index of biodiversity.

Figure 3: sea bream gut bacterial community composition at phylum (a) and family levels (b).

Figure 4: weighted and unweighted UniFrac distance PCoA of the gut bacterial community of sea bream treated with different diets, color code: S30 diet red, S20 diet green, S10 diet yellow, S0 diet blue, C diet purple. MDS1 and MDS1 represent the 15.4 and 2.6 % of the total variability, respectively. Permutation test with pseudo F-ratios: $P = 0.107$ and $P = 0.091$ for weighted and unweighted UniFrac, respectively.

Figure 5: relative abundance of bacteria composition per sample at phylum (a) and family levels (b).

Figure 6: box plot showing the relative abundance of (a) *Cyanobacteria*, (b) *Synergistetes*, (c) *Actinobacteria* and (d)

887 *Lactobacillaceae* in different diets. Significance of the
888 differences was obtained by Kruskall-Wallis test.
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Table 1. Formulation and proximate composition of the experimental diets

Ingredients (g kg^{-1})	S0	S10	S20	S30	C
FM North Atlantic	150	150	150	150	350
Hi Pro SBM	0	100	200	300	0
Wheat meal	206.4	165.6	125.8	84.0	229.3
Wheat gluten	226	199.1	175.9	150	127.7
Corn gluten	200	185	165	150	130
Sunflower meal	80	60	40	20	40
Fish oil North Atlantic	132.5	135.3	138.3	141	118
Vit/Min premix*	5	5	5	5	5
Proximate composition (g kg^{-1})					
Moisture	77	76	78	80	60
Crude protein	466	466	479	478	460
Crude fat	194	192	199	209	197
Ash	45	47	48	57	69

FM, fishmeal; SBM, soybean meal; S0, 0 g kg^{-1} SBM diet; S10, 100 g kg^{-1} SBM diet; S20, 200 g kg^{-1} SBM diet; S30, 300 g kg^{-1} SBM diet; C, control diet.
*Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011).

Table 2. Growth performance of sea bream fed the experimental diets

	Experimental diet				
	S0	S10	S20	S30	C
Growth					
IBW (g)	76.0 ± 1.6	75.1 ± 0.6	77.1 ± 3.1	76.7 ± 1.9	74.4 ± 0.9
FBW (g)	249.1 ± 6.1	249.2 ± 3.9	257.6 ± 6.2	259.5 ± 5.9	256.2 ± 5.8
SGR (day^{-1})	1.17 ± 0.03^{a}	1.20 ± 0.01^{ab}	1.22 ± 0.01^{ab}	1.25 ± 0.01^{b}	1.21 ± 0.04^{ab}
FI (% day^{-1})	1.40 ± 0.01^{a}	1.45 ± 0.01^{ab}	1.44 ± 0.03^{ab}	1.51 ± 0.03^{c}	1.46 ± 0.02^{bc}
FCR	1.33 ± 0.03	1.35 ± 0.01	1.33 ± 0.01	1.36 ± 0.04	1.36 ± 0.05

S0, 0 g kg^{-1} soybean meal SBM diet; S10, 100 g kg^{-1} SBM diet; S20, 200 g kg^{-1} SBM diet; S30, 300 g kg^{-1} SBM diet; C, control diet. IBW, initial body weight; FBW, final body weight; SGR, specific growth rate, 100 * (ln FBW − ln IBW) / days; FI, feed intake, 100 * (crude feed intake / ((FBW + IBW) / 2) / days; FCR, feed conversion rate, (feed intake / weight gain).

Data are given as the mean (n=3; n=60 for IBW and FBW) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

Table 3. Viscerosomatic index, hepatosomatic index, body composition and nutritional indices of sea bream fed the experimental diets.

Experimental diet					
	S0	S10	S20	S30	C
VSI	5.62 ± 0.83	5.98 ± 0.95	5.99 ± 0.93	5.72 ± 0.70	5.78 ± 1.03
HSI	1.70 ± 0.34	1.60 ± 0.31	1.64 ± 0.33	1.59 ± 0.32	1.59 ± 0.35
Whole body composition (g kg^{-1})					
Moisture	619 ± 4.7	626 ± 6.2	628 ± 4.6	632 ±1.3	615 ± 5.1
Crude protein	174 ± 2.4	174 ± 2.6	175 ± 2.6	179 ± 0.9	173 ± 0.7
Total lipids	173 ± 9.3	175 ± 7.1	175 ± 9.2	180 ± 5.6	174 ± 6.0
Ash	33 ± 2.1	33 ± 1.3	32 ± 2.5	30 ± 0.7	33 ± 2.0
Nutritional indices					
PER	1.62 ± 0.04	1.59 ± 0.01	1.58 ± 0.03	1.54 ± 0.04	1.60 ± 0.06
GPE	28.8 ± 0.92	28.2 ± 0.50	28.4 ± 1.07	28.3 ± 0.86	28.2 ± 1.21
GLE	69.6 ± 4.14	70.1 ± 3.98	69.8 ± 5.26	67.2 ± 4.10	70.3 ± 4.92

S0, 0 g kg^{-1} soybean meal SBM diet; S10, 100 g kg^{-1} SBM diet; S20, 200 g kg^{-1} SBM diet; S30, 300 g kg^{-1} SBM diet; C, control diet. VSI, viscerosomatic index; HSI, hepatosomatic index; PER, protein efficiency ratio; GPE, gross protein efficiency; GLE, gross lipid efficiency.

Data are given as the mean (n=3; n=15 for VSI and HSI) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). PER, ((final body weight − initial body weight) / protein intake); GPE, (100*[(% final body protein * final body weight) − (% initial body protein *initial body weight)] / total protein intake $fish^{-1}$); GLE, (100*[(% final body lipid * final body weight) − (% initial body lipid * initial body weight)] / total lipid intake $fish^{-1}$).

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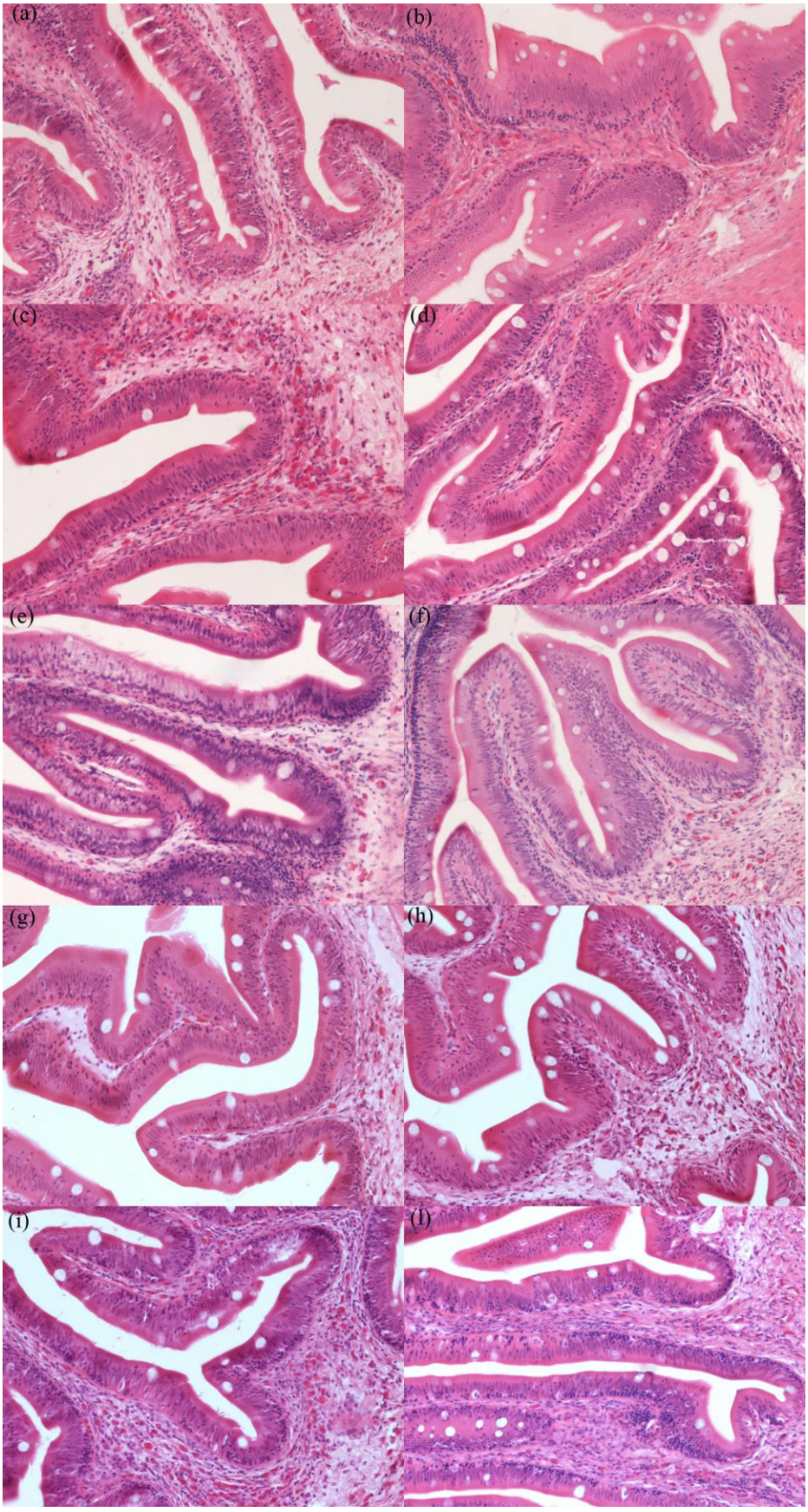
941 Figure 1



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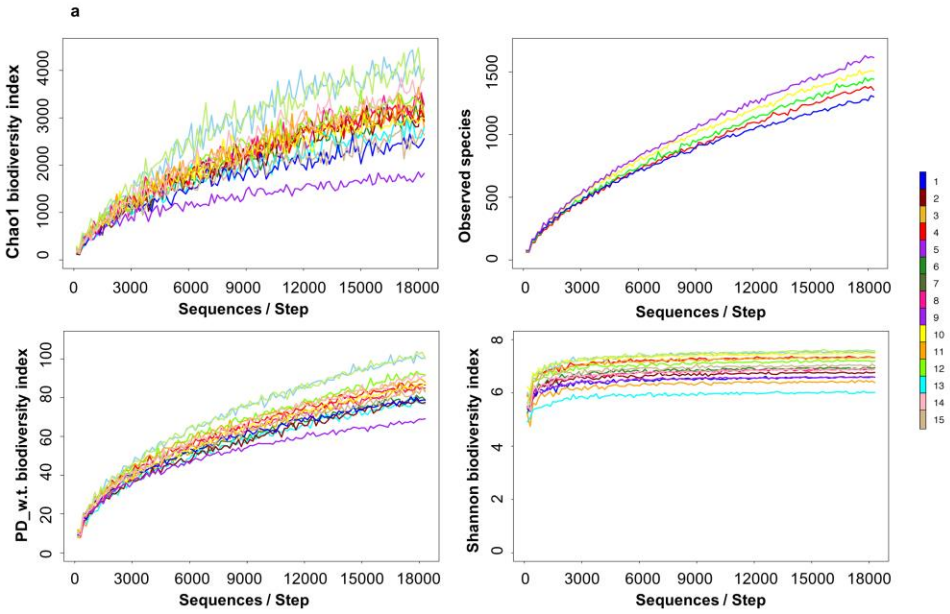

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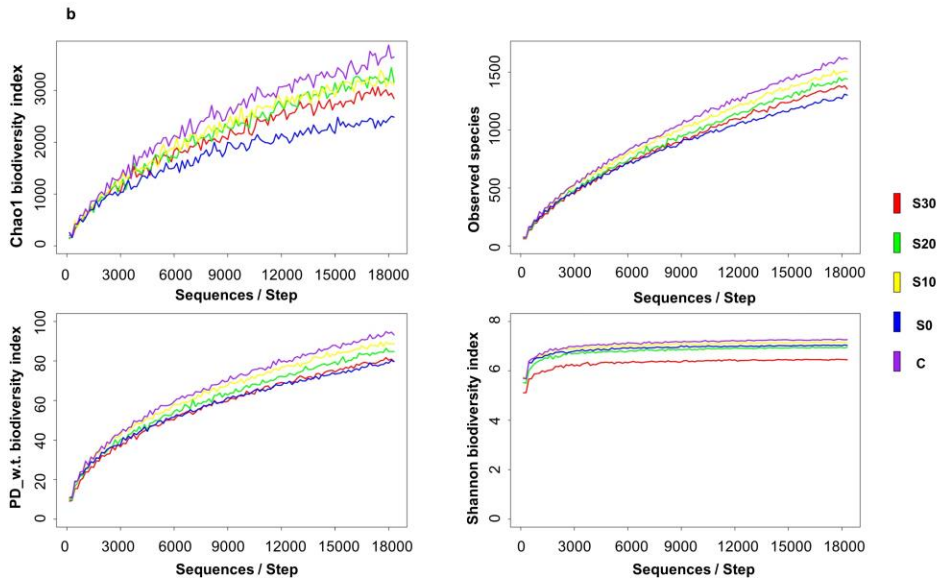

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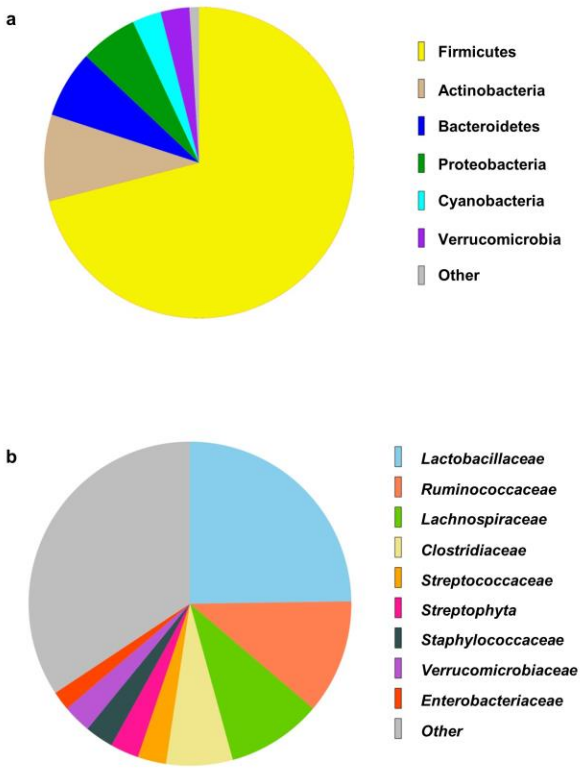

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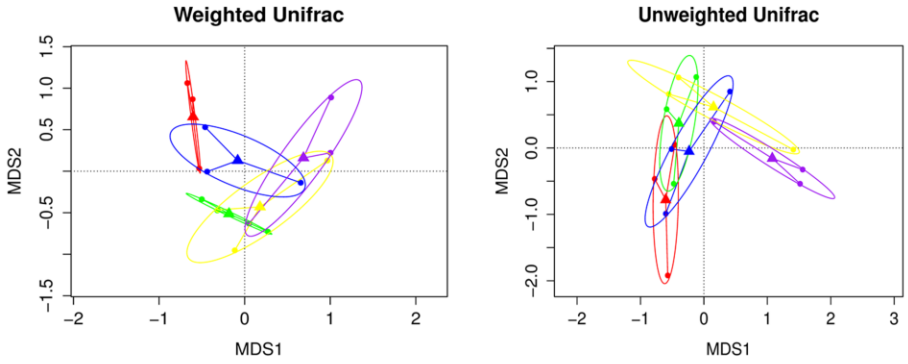

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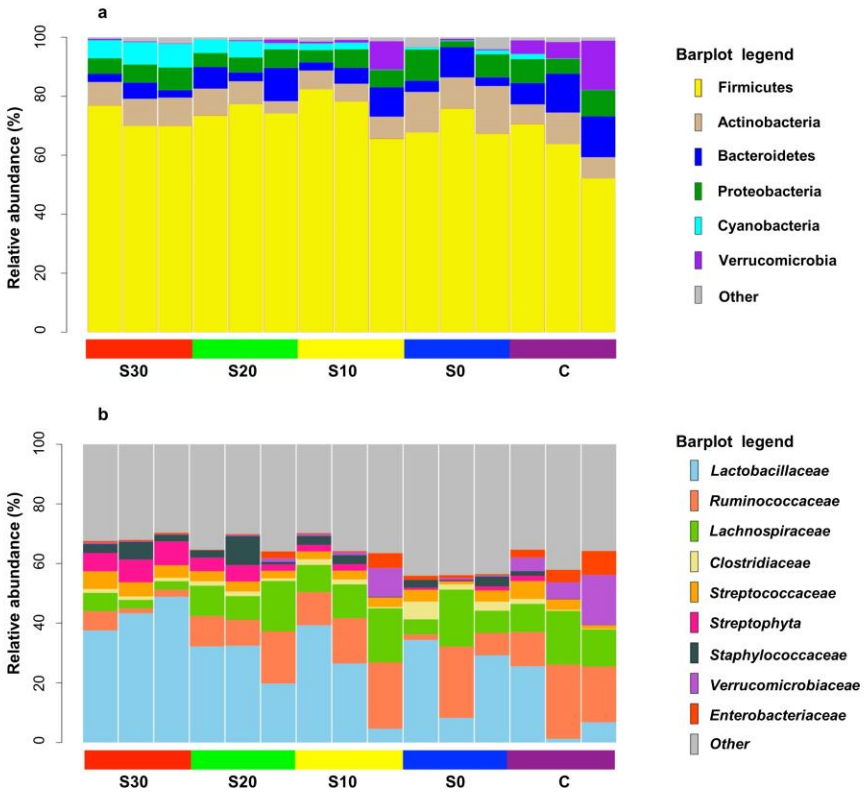

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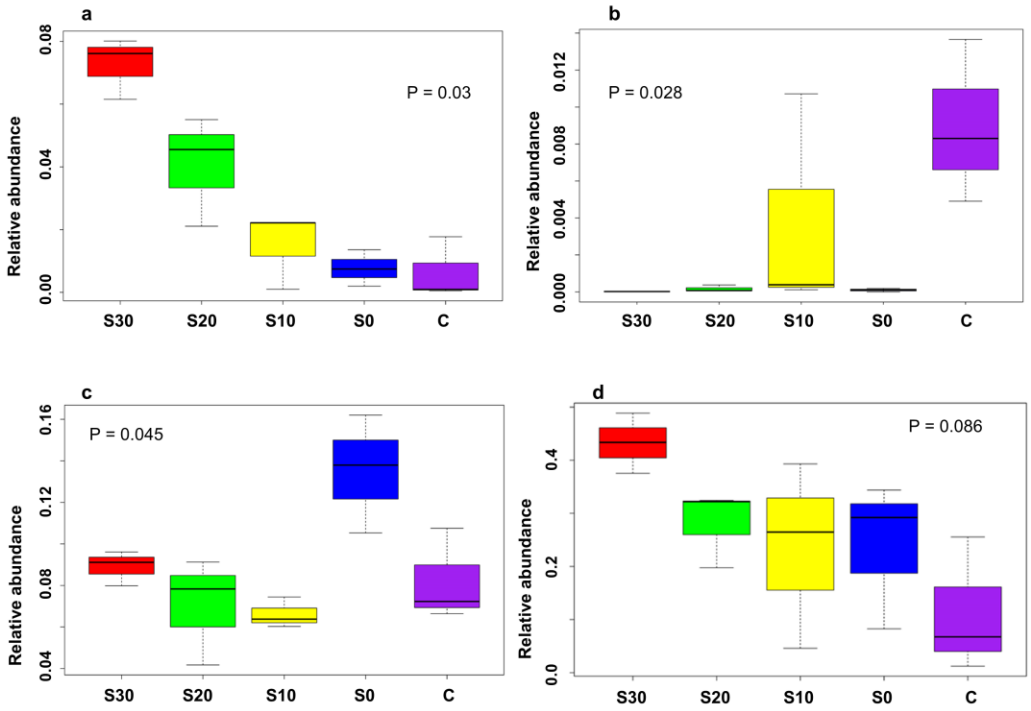
960 Figure 6



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