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**Feeding gilthead sea bream with increasing dietary bacterial single cell protein level:
implication on growth, plasma biochemistry, gut histology, and gut microbiota**

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Abstract

Bacterial single cell protein (SCP) is considered a promising circular protein ingredient for aquafeed, due to the high protein content, and for the possibilities to grow them on different substrates such as organic waste thus leading to low environmental footprint and affordable production costs. Their use as raw material has been assessed in several farmed species, however, research on Mediterranean ones is still scarce. Hence, a study was undertaken to evaluate growth, plasma biochemistry, gut histology and gut microbiota (GM) response of gilthead sea bream (*Sparus aurata*) fed diets with increasing levels of bacterial SCP in comparison to a control without SCP. Three isonitrogenous and isolipidic extruded diets (44% protein; 19% lipid) were formulated with different bacterial SCP (derived from *Corinebacterium glutamicum*) level (10% SCP, SPC10; 15%

SCP, SCP15; 20% SCP, SCP20) to replace vegetable protein ingredients (total replacement of soy protein concentrate and partial replacement of corn gluten), while a control diet (CTRL) was formulated without SCP. Fish groups of 45 individuals (initial weight: 75 g) were fed to visual satiation over 108 days. At the end of the trial there were no significant differences on growth, feed intake, feed conversion rate, protein efficiency ratio and protein apparent digestibility. Most of plasma parameters were found to be equal for all treatments, except for those related to nucleic acids molecules degradation such as phosphorus and urea which were higher in SCP10 and SCP20 compared to CTRL, respectively. No morphological alterations were found in the intestines of any fish analysed. Different responses of the overall GM structure in relation to the bacterial SCP inclusion level were detected. Specifically, SCP exerted a positive effect on GM internal diversity which increased at increasing dietary SCP inclusion level. In addition, SCP inclusion lead to increase in the abundance of *Bacillus* spp, taxa which can potentially support nutrition, immune system, and disease resistance. In conclusion, it seems feasible to include up to 20% of SCP dietary level for gilthead sea bream without compromising growth, feed efficiency and health parameters.

Keywords

Single cell protein, gilthead sea bream, growth, plasma biochemistry, gut microbiota

Introduction

Single cell protein (SCP) are dehydrated cells of unicellular organisms such as fungi, bacteria, yeast and microalgae deriving from fermentation processes of biomass from industry and agriculture (i.e. molasses, whey, starch, alkanes, hydrocarbons, celluloses, ammonia, nitrate, natural gas) (Sharif et al., 2021). SCP are used for animal feed due to many factors: high growth rate of microorganisms, the wide variety of substrates they can metabolize, the low processing costs, and the production of derived nutrients and functional molecules. They are mainly used in human or animal nutrition as protein sources, due to high protein content (60–82% on dry matter), with a suitable amino acid profile. In addition, the content of beneficial lipids, carbohydrates, vitamins and minerals may promote their higher nutritional values in comparison to conventional protein sources (Zepka et al., 2010; Aruna et al., 2017; Sharif et al., 2021). Moreover SCP also requires low water demand compared to the plant sources and are not affected by environmental conditions (Sharif et al., 2021). In aquaculture, SCP has been considered as a protein replacement to standard protein commodities (i.e. fishmeal, FM and soy products) and most of the studies carried out have shown positive impacts on increasing growth (Guo et al., 2019).

Yeast SCPs, are a protein source rich in vitamins and micronutrients which can provide several benefits such as enhancing the immune response, reducing stress, and modulating gut microbiota (Rawling et al., 2019; Rimoldi et al., 2020; Ciji and Akhtar, 2021). These effects were tested on several fish species, such as Atlantic salmon, *Salmo salar* (Hansen et al., 2021), rainbow trout, *Oncorhynchus mykiss*, gilthead seabream *Sparus aurata*, and European seabass *Dicentrarchus labrax* (Agboola et al., 2021).

Microalgae SCP are a good crude protein source, (60 %) but is mostly used for production of omega-3 fatty acids (EPA and DHA) and carotenoids (Glencross et al., 2020). Many studies have been conducted on the health, immune response and digestibility of microalgae SCP of several species such as rainbow trout (Zhang et al., 2020), Atlantic salmon (Hart et al., 2021), gilthead sea bream (Carvalho et al., 2020), and European sea bass (Messina et al., 2019).

Bacteria SCP are considered to be a promising protein source for aquafeed, due to its ability to alter their composition according to different production setting. They have a high content of raw proteins (80%), high growth rate, and they are able to grow on different substrates such as organic waste and petrochemicals i.e. ethanol, methane, methanol and nitrogen, syngas, CO₂ and H₂ (Delamare-Deboutteville et al., 2019; Jones et al., 2020). Among the other previously mentioned SCP sources, minor attention has been devoted to exploring the use of bacterial SCP as an aquafeed ingredient. Some studies were performed on Atlantic salmon (Aas et al., 2006), rainbow trout (Hardy et al., 2018), Nile tilapia *Oreochromis niloticus* (Smarason et al., 2019), Japanese yellowtail *Seriola quinqueradiata* (Biswas et al., 2020), and African catfish *Clarias gariepinus* (Adeoye et al., 2021). Most of them highlight the possibility to replace 5-20% FM in rainbow trout, 30% FM in catfish, 4-36% FM in Atlantic salmon, and 50% FM in Nile tilapia. To the best of our knowledge, only one study reported the application of dietary bacterial SCP in Mediterranean fish species (Solé-Jiménez et al. 2021). The authors were able to successfully replace 50% of FM using a commercial protein source made of bacterial and processed animal proteins without compromising growth performance of gilthead seabream.

In recent years, in the aquaculture sector significant amounts of dietary FM have been successfully replaced with alternative ingredients mainly derived from commodity agricultural crops proteins such as plant-based proteins including various legumes such as soya bean meal. As consequence, the increasing demand for plant proteins for animal feed production, has been also associated to environmental impact concerns over deforestation, land-use displacement and eutrophication (Woodgate et al., 2022).

The aim of this study was to explore the efficacy of dietary inclusion level of bacterial SCP to replace plant protein sources. Growth, plasma biochemistry, gut histology, and gut microbiota during the on-growing of gilthead sea bream are investigated.

Materials and methods

2.1 Experimental diets

Three diets (44% protein; 19% lipid) were formulated to contain increasing level of single cell protein (SCP, Gordini srl, Italy) from *Corinebacterium glutamicum* (10% SCP SPC10, 15% SCP SCP15, 20% SCP SCP20) in order to replace vegetable ingredients (total replacement of soy protein concentrate and partial replacement of corn gluten) while a control diet (CTRL) without SCP and containing the same amount of FM was used (Table 1). Diets were formulated with FM and a mixture of vegetable ingredients currently used for sea bream in aquafeed (Parma et al., 2016). The diets were produced via extrusion (pellet size = 3.0 mm) by SPAROS Lda (Portugal). Proximate composition of the diets and amino acid composition of the SCP are reported in Tables 1-2.

2.2 Fish and experimental conditions

A growth trial was performed at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna (Cesenatico, Italy). Gilthead sea breams were obtained from an Italian hatchery and acclimatized to the facilities for 7 days before the beginning of the trial. Forty-five fish per tank were randomly distributed in twelve 500 L tanks. Each diet was administered to triplicate tanks over 108 days. Tanks were provided with natural seawater and connected to a closed recirculating system (overall water volume: 7000 L; Oxygen level $8.0 \pm 1.0 \text{ mg L}^{-1}$; Temperature $23 \pm 1.0 \text{ }^{\circ}\text{C}$, Salinity 25 g L^{-1} , pH 7.8-8.0) according to Busti et al. (2020a). Fish were hand fed to visual satiation twice a day (8:30, 16:00) for six days a week. Feeding procedures were made to prevent any feed losses, however in cases of uneaten feed, pellets were collected, dried overnight at 105°C , and weighted for overall calculation.

2.3 Sampling

Before each sampling procedures fish were anaesthetized (100 mg L^{-1}) or euthanised (300 mg L^{-1}) by tricaine methanesulfonate MS-222 (Sigma-Aldrich). At the beginning and the end of the trial individual fish weight was measured in each tank. The approximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 15 fish and on a pooled sample of 5 fish per tank at the end of the trial. Blood from 5 fish per tank ($n=15$ fish per diet treatment) was collected at the end of the trial from the caudal vein. Samples were then centrifuged ($3000 \times g$, 10 min, 4°C) and plasma aliquots were stored at -80°C until analysis (Bonvini et al., 2018). On the same specimens, three fish (12 fish for each experimental group) were used for histology. For

each fish, the gut was gently removed from the celom cavity and the anterior and middle part of the intestine were fixed in buffered formalin and then processed for morphological evaluation. Digesta content (n=5 fish per tank) from distal intestine was also individually sampled at the end of the trial and immediately stored at -80°C for gut microbiota investigation according to Parma et al. (2020).

2.4 Digestibility assessment

After the end of the trial, the remaining groups of fish were used to determine the apparent digestibility coefficient (ADC) of dry matter and protein, by the indirect method with diets containing yttrium oxide according to Busti et al. (2020b). Fish were fed according to the different diets and after that at 8 h post-prandial fish were euthanised by overdose of anaesthetic and faeces were collected after fish dissection and stripping distal intestine. Faeces were then pooled (N=3) for each tank and kept at -20°C until analysis for yttrium, dry matter and protein. ADC was calculated as follows: $\text{ADC} = 100 * (1 - (\text{dietary Y2O2 level} / \text{faecal Y2O2 level})) * ((\text{faecal nutrient} / \text{dietary nutrient}))$.

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

2.5 Gut histology

For each sample, 24 intestinal paraffin sections (both anterior and middle part) were obtained. To avoid morphometric evaluations in serial sections and, consequently, to

analyse the same characteristics, the first, sixth, eighteenth and twenty-fourth sections were processed and stained with haematoxylin and eosin. Since the mucosa of the anterior intestinal tract showed very complex and branching folds, it was decided to evaluate the absorbent surface using the binarization method. Binarization was done in a blind fashion by 2 expert investigators. The intestinal sections were scanned with the Nikon DS-Qi1Nc digital camera at 10X magnification, using NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands) with an interactive tool, Scan Large Image, suitable for subsequent image analysis. This tool acquires an image with an area of interest that exceeds the camera's field of view, capturing a large image made up of multiple image frames stitched by an automatic algorithm that cannot be loaded in one piece. Automated Image Binarization was applied to area of each selected intestinal image by means of the software NIS Elements software BR 4.20.01. Image Binarization is a widely used method that allow distinguishing objects of interest from background. Indeed, determines a grey threshold and assigns each pixel of a digital image to one class (image objects) if its grey value is greater than the determined threshold and otherwise to the other class (image background). Specifying correct threshold limits is a crucial procedure of the automated image analysis. The point is to determine which pixels will and which will not be included in the binary layer and thereby distinguish objects to be analyzed from background. By threshold, its possible highlighted the absorbent surface of the intestinal tract. In our case, using binarization we were able to separate the pixels, which represent absorbent surface (brighter pixels) from those, that represent the rest of the layers of the intestinal sections (Fig. 1A and B). The area of measurement can be restricted by a user-defined region of interest ROI (Fig. 1C). ROI is a strong tool used mainly to measure varying image intensity inside the ROIs or number of binary objects

inside each ROI, Object Count to restrict binary objects to areas of interest only (Fig. 1C and D). Consequently, this allowed us to quantify the cellular absorbent surface covered by villi. The measured obtained were expressed in square millimetres.

2.6 Plasma biochemistry

Glucose (GLU), urea, creatine, uric acid, total bilirubin, cholesterol (CHOL), triglycerides (TRIG), high density lipoprotein (HDL), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), lactate (LAC), calcium (Ca²⁺), phosphorus (P), potassium (K⁺) sodium (Na⁺), iron (Fe), chloride (Cl), magnesium (Mg) were determined in the plasma using samples on an automated analyser (AU 480; Olympus/Beckman Coulter, Brea, CA, United State). OSR (Olympus system reagent) method was utilized to evaluate the reported variables according to Pelusio et al. (2021). The ALB/globulin (GLOB), Na/K ratio and Ca x P were calculated.

2.7 Calculations

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

(%) = $100 * [(\% \text{ final body protein} * \text{FBW}) - (\% \text{ initial body protein} * \text{IBW})] / \text{total protein intake fish}$. Economic conversion ratio (ECR) ($\text{€}/\text{kg fish}^{-1}$) = FCR * feed cost

2.8 Proximate composition analysis

Diets and whole body of sampled fish were analysed for an approximate composition as reported in Parma et al. (2020). In brief, the moisture content was obtained by observing the weight loss after drying the 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.

2.9 Gut Bacterial Community DNA Extraction and Sequencing

Total DNA was extracted and analysed from individual distal intestine content obtained from 5 fish per tank (300 mg per fish) at the end of the trial, as previously reported in Pelusio et al. (2021) and from 4 samples of the different diet using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). DNA was quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at -20 °C until further processing. To target the transient bacterial community, the amplification of the V3-V4 hypervariable regions of the 16S rRNA gene was carried out using the 341F and 785R primers carrying Illumina overhang sequencing adapters and 2 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems). The thermal cycle was performed as already described

by Pelusio et al. (2021) using 30 amplification cycle. PCR products were purified, and indexed libraries were prepared following Illumina protocol “16S Metagenomic Sequencing Library Preparation”. Libraries were normalized to 4 nM and pooled. Pooled libraries were denatured with 0.2 N NaOH and diluted to 6 pM with 20% PhiX control. Sequencing was performed on Illumina MiSeq platform using 2 x 250 bp paired-end protocol according to the manufacturer’s instructions (Illumina, San Diego, CA). At the end of the sequencing process, raw sequences were processed combining PANDAseq and QIIME2 pipelines (Bolyen et al., 2019; <https://qiime2.org>). High-quality reads, obtained after a filtering step for length (minimum/maximum = 350/550 bp) and quality with default parameters, were cleaned using DADA2 (Callahan et al., 2016) and clustered into amplicon sequence variants (ASVs) using VSEARCH algorithm (Rognes et al., 2016). Taxonomy was assigned using RDP classifier against SILVA database (Quast et al., 2013). The 4 feed samples were discarded for subsequent analysis due to the high number of “unassigned taxa”. Three different metrics were used to evaluate internal ecosystem diversity (alpha-diversity) – Faith’s Phylogenetic Diversity (PD_whole_tree), Chao1 index for microbial richness, and number of observed ASVs. UniFrac distances were computed to estimate inter-sample ecosystem diversity (beta-diversity) and used as input for Principal Coordinates Analysis (PCoA).

2.10 Statistical analysis

All data are presented as mean \pm standard deviation (SD). A tank was used as the experimental unit for analysing growth performance and a pool of five fish were considered the experimental unit for analysing carcass composition. Individual fish were

used for analysing plasma biochemistry. Data on growth, nutritional indices, apparent digestibility, plasma biochemistry were analysed by a one-way analysis of variance (ANOVA) with Tukey's post hoc test. The normality and/or homogeneity of variance assumptions were validated for all data preceding ANOVA. The gut morphometric evaluation was expressed as mean \pm SD. The data obtained was analysed by *t*-test. The differences among treatments were considered significant at $p \leq 0.05$. All microbiota analysis and respective plots were produced using R software (<https://www.r-project.org/>) with “vegan” (<http://www.cran.r-project.org/package=vegan/>), “Made4” (Culhane et al., 2005) and “stats” packages (<https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html>). Data separation was tested by a permutation test with pseudo-F ratios (function “Adonis” in “vegan” package). When required, Wilcoxon and Kruskal–Wallis test were used to assess significant differences in alpha diversity and taxon relative abundance between groups. *P*-values were adjusted for multiple comparisons using the false discovery rate (FDR) (function *p.adjust* in the “stats” package), and a *p*-value ≤ 0.05 was considered statistically significant, while a *p*-value between 0.05 and 0.1 was considered as a trend.

3. Results

3.1 Growth, nutritional indices, and protein digestibility

Growth performance parameters are reported in Table 3. No significant differences on FBW, weight gain and SGR were detected between dietary treatments. Similarly, no significant differences on FI, FCR, ECR and survival were also observed (Table 3).

Data on body composition, nutritional indices and apparent digestibility are shown in Table 4. Protein body content was lower in the SCP15 diet compared to the CTRL diet while no differences on moisture, lipid and ash content were detected between treatments. GPE was lower in SCP15 compared to the CTRL diet while no significant differences on PER and apparent digestibility of dry matter and protein was detected. Concerning somatic indices, CF was lower in the SCP20 diet compared to SCP10, while no significant differences between treatments were detected for HSI and VSI.

3.2 Gut histology

Anterior and middle intestine were lined by a tunica mucosa constituted by epithelium and lamina propria forming folds/villi along all tracts. The intestinal mucosa of the anterior intestine tract was organized in mucosal folds formed by tall primary everting constituted by the mucosa and submucosa: from their main axis, secondary everting of epithelium and *lamina propria* formed other folds/villi-like. Along the entire length of the middle intestine, the complex folds were rare/absent than to anterior tract: most of these folds resemble normal villi. No inflammation features were observed, such as villi shortening and nuclear positioning disparity. Regarding morphometric analysis no significant differences in both anterior and middle intestinal tracts were observed (Table 5).

3.3 Plasma biochemistry

Plasma parameters are shown in Table 6. Urea was higher in SCP20 compared to the other groups. TRIG were higher in SCP15 compared to CTRL. HDL was higher in SCP20 compared to CTRL. Ca^{+2} and Cur Ca^{+2} were significantly higher in the SCP20 diet than in the CTRL diet, while P and CaxP were higher in SCP10 compared to CTRL. No significant differences among treatments were detected for GLU, CREA, Uric Ac, Tot Bil, CHOL, TP, ALB, AST, ALT, ALP, CK, LDH, LACT, K^+ , Na^+ , Fe, Cl, Mg, ALB/GLO, Na/K.

3.4 Faecal bacterial community profiles related to dietary groups

The 16S rRNA gene sequencing was performed on a total of 60 distal intestine content samples, yielding 401'841 high-quality reads (mean \pm SD, 6'182 \pm 2'574) and clustered into a total of 6'252 ASVs. To assess whether the treatments with increasing SCP could exert an effect on the gut bacteria community during the growth process of gilthead seabream, the gut microbiota (GM) was analysed for each dietary group at the end of the trial. The GM variations between samples (beta-diversity) were assessed by the Principal Coordinates Analysis (PCoA) based on Unweighted UniFrac distances, with the taxa most explaining sample segregation being superimposed on the bidimensional space. In addition, the gut microbial community internal diversity was represented with three different metrics for each dietary group: PD_whole_tree, Chao1 index, and observed_ASVs. According to the findings (Figure 2A), all SCP groups showed a significant variation compared to the control group, in terms of overall GM composition ("pairwise Adonis permutation test", $p < 0.01$). Focusing on dietary group, a significant overall GM composition variation between all dietary groups was observed (SCP10 vs

SCP15, SCP10 vs SCP20, SCP 15 vs SCP20; “pairwise Adonis permutation test”, $p < 0.01$). As for internal ecosystem diversity, SCP10 dietary group showed a significant reduction of the alpha-diversity compared to control group in all the 3 metrics considered (SCP10 vs CTRL; Wilcoxon rank-sum test, $p = 0.005$) (Figure 2B). The SCP15 group showed a significant reduction of the microbial internal ecosystem diversity compared to control group only when considering PD_whole_tree metric, (SCP15 vs CTRL; Wilcoxon rank-sum test, $p = 0.007$) (Figure 2B). On the contrary, SCP20 group showed a significant increase of alpha-diversity, as for chao1 index and observed_ASVs metrics, when compared to the control group (SCP20 vs CTRL; Wilcoxon rank-sum test, $p < 0.05$) (Figure 2B). When considering only the different dietary groups, a significant increase of microbial internal ecosystem diversity was observed associated with the increase of SCP concentration, in chao1 index and observed_ASVs metrics (Figure 2B) (SCP10 vs SCP15, SCP10 vs SCP20, SCP 15 vs SCP20; Wilcoxon rank-sum test, $p = 0.005$, $p = 0.0002$, $p = 0.02$, respectively). To further assess the GM composition of gilthead sea bream fed with different SCP concentrations, the overall composition at different phylogenetic levels was investigated, as reported in Figure 3: at phylum (Figure 3A) and family level (Figure 3B). More specifically, the most abundant phylum and the most represented families in the gilthead sea bream GM of the 4 experimental groups were showed in the Table 7. Moving to a lower taxonomic level, some compositional differences were observed at genus level among dietary groups (Wilcoxon rank-sum test $p < 0.05$) (Figure 4). According to the data, the relative abundance of *Bacillus*, *Escherichia-shigella* and *Oceanobacillus* genera was significantly lower in CTRL group compared to fish fed with different SCP concentrations (Wilcoxon $p < 0.05$). On the other hand, the relative abundance of *[Eubacterium] coprostanoligenes* group, *Weissella*,

Ruminococcaceae UCG-10, *Ruminococcaceae* UCG-013, *Ruminococcaceae* UCG-014, *Ruminococcaceae* UCG-005, *Christensenellaceae* R-7 group genera was generally significant lower in fish fed with different SCP concentrations compared to control group (Wilcoxon $p < 0.05$). With a focus on *Staphylococcus* genus, a direct effect of the SCP concentration on its relative abundance was observed. More specifically, a significantly higher abundance of *Staphylococcus* genus in fish fed with SCP10 diet compared to control group (Wilcoxon, $p = 0.029$) was reported. Higher SCP concentrations were instead associated with a significant reduction of the relative abundance of this genus compared to SCP10 group, thus showing a significant relative abundance reduction in fish fed with SCP15 diet compared to SCP10 diet (Wilcoxon, $p = 0.04$). SCP20 group showed a significant reduction of the relative abundance of *Staphylococcus* genus compared to both SCP15 and SCP10 groups (Wilcoxon, $p = 0.0004$, $p = 0.0005$, respectively) and a tendency in abundance reduction compared to CTRL group (Wilcoxon, $p = 0.09$) (Figure 4)

Discussion

The growth parameters (FBW, WG, SGR, FCR and FI) observed during the trial, showed similar results regardless the level of SCP. Focusing on the feed intake, the absence of significant differences has a positive implication considering its correlation with appetite and palatability of feed. Most of the substances that increase the attractiveness and palatability of feed in fish are characterized by low molecular weight including nitrogenous and amphoteric components, amino acids, betaines, and nucleotides. High nucleotide content derived from bacterial SCP could enhance

palatability and feed intake. It is recognized that nucleotides may act as a taste enhancer specifically due to inosine and inosine monophosphate which were identified as a feeding stimulant (Gamboa-Delgado et al., 2018; Hossain et al., 2020). This could be particularly relevant when high level of plant protein sources is used to replace FM since they contain significantly less nucleotides and the present of plant anti-nutritional factors may interfere with palatability. Our results are in contrast with previous studies conducted on rainbow trout (Hardy et al., 2018) and black sea bass (Chen et al., 2020), proving that the replacement of soy protein with bacterial SCP could reduce FI due to low palatability. The authors postulated that the source of bacterial protein meal and the process by which it was dried or the presence of flavour compounds could have been responsible for low palatability. The absence of statistical difference on final body weight, weight gain and SGR, suggests that the inclusion of bacterial SCP to up to 20% could replace soy protein concentrate and corn gluten without affecting growth performance. In Atlantic salmon and rainbow trout, up to 30% replacement of soy protein with SCP did not affect animal growth (Romarheim et al., 2011; Hardy et al., 2018). This is in accordance with a study conducted on Nile Tilapia by Maulu et al. (2021), providing that dietary increasing level of *Clostridium autoethanogenum* protein could replace up to 20% of soybean meal improving growth performance. However, lower body protein content was observed in SCP15. Lower protein content in SCP15 was also reflected in the lower values achieved for protein efficiency (GPE) in the same treatment. However, differences in protein efficiency were minimal and were not reflected in the FCR obtained. Overall, the absence of growth differences, FCR, protein digestibility, and PER, suggest an optimal digestibility, nutrients absorption, and utilization of bacterial SCP in gilthead sea bream up to 20% of dietary inclusion level. Concerning somatic indices, no differences were

evaluated in HSI and VSI, assuming no effect on assimilation and distribution of nutrients within the animal body's tissues.

Most of the plasma biochemistry results, such as TP, ALB, Glu, TC, and ALT, did not show any statistical differences, demonstrating that SCP could guarantee optimal nutrition and general health status (including liver health status as indicated by AST and ALT) in agreement with previous plasma values on this species and with the results obtained in Nile Tilapia fed bacterial SCP (Parma et al., 2020; Maulu et al., 2021). However, Ca^{+2} and CurCa^{+2} values were higher in SCP20 and, CaxP was higher in SCP10 compared to control diet. Differences when compared to the standard calcium values could represent a stress indicator, causing imbalance plasma ion level and increasing plasma osmolality (Mancera et al., 2002; Mateus et al., 2017). Despite this, calcium values shown in this work, are in line with previous works of the same species and within values of healthy fish (Peres et al., 2013; Pelusio et al., 2021). It is also worth highlighting the higher value of plasmatic urea in SCP20. In agreement with Oliva-Teles et al. (2006) high levels of SCP can lead to high levels of non-protein nitrogen content, such as nucleic acid, mostly represented by RNA. Ammonia is the final product of pyrimidines catabolism, which are one of the main components of RNA molecules. Even the higher values of inorganic phosphorus detecting in SCP10 could be related to the degradation of RNA molecules, even if this trend was not observed at the higher inclusion level (SCP15, SCP20) tested. Plasma triglyceride and HDL were higher in the fish fed SCP15 and SCP20 compared with the control diet, respectively. According to Maulu et al. (2021) the inclusion of 200g kg of *Clostridium autoethanogenum* bacterial SCP increased plasma triglycerides and cholesterol in Nile Tilapia. Although it is not clear how SCPs could affect this process, the authors suggested an improvement in lipid metabolism and a role

of bacterial SCP in glucolipid metabolism to maintain whole-body energy homeostasis through the adenosine monophosphate-activated protein kinase (AMPK) signalling pathway. Interestingly, dietary nucleotide inclusion also increased blood triglycerides in red sea bream *Pagrus major*, but further research is needed to illustrate lipid transport in fish administered with nucleotides (Hossain et al., 2016a; Hossain et al., 2020).

Histology is considered a valid method for evaluating aquafeed ingredients since several raw materials (mainly of vegetable origin) are known to induce morphological changes thus altering the processes of nutrient digestion, absorption and pathogen resistance (Rey et al., 2020). Gut histology revealed no histopathological changes of SCP in the intestines of the animals examined indicating that SCP could replace soy derived proteins, without altering the anatomic structure of the intestine. The literature is lacking regarding the effects of bacterial SCP-enriched diets on gut histology in fish species. However, dietary nucleic acid supplementation has a positive influence on intestinal morphology such as increased enterocyte height and compensatory of intestinal morphology damage. This is due to high inclusion of alternative vegetal protein that were reported in several fish species (Hossain et al., 2016b; Hossain et al., 2020). It is worth to mention that the level of soybean meal used in the present study in all the treatments was within standard practical levels, which are known to not induce intestinal inflammatory process in this species, as reported by Bonaldo et al. (2008) and Parma et al. (2016).

A growing number of researchers have addressed the study of gut microbiota in fish species of commercial interest, since it is recognized as a powerful tool for assessing digestive condition and gut health. In gilthead sea bream the inclusion of different protein ingredients such as soy, insect, yeast and eggs peptide, have recently shown potential for a GM reconfiguration (Parma et al., 2016; Antonopoulou et al., 2019; Rimoldi et al., 2020;

474 Naya-Català et al., 2021a, 2021b). According to our findings, GM was dominated by
475 Firmicutes, at phylum level while *Staphylococcaceae*, *Bacillaceae*, *Lactobacillaceae*,
476 *Leuconostocaceae* were the most represented taxa at family level. These data are in
477 agreement with previous findings on faecal GM of gilthead sea bream fed practical
478 aquafeed ingredients. Firmicutes and lactic acid bacteria (LAB) have been associated to
479 vegetal ingredients and considered a beneficial taxa able to promote nutrient digestion
480 and counteract pathogen invasion (Parma et al., 2016; Parma et al., 2020; Panteli et al.,
481 2021). Different responses of the overall GM structure in relation to the bacterial SCP
482 inclusion level were detected as evidenced by a significant separation in the PCOA
483 analyses of all the SCP inclusion level compared to the control diet. In addition, dietary
484 inclusion level exerted an effect on GM internal diversity which increased along with
485 higher SCP levels. In agreement with our findings, in gilthead sea bream the replacement
486 of 50 and 100% of FM with a mix of processed animal proteins (PAPs) and bacterial SCP,
487 leads to increased in alpha diversity indexes (Solé-Jiménez et al., 2021). Increase in GM
488 diversity may have positive implications for gut health due to increased competition
489 against opportunistic pathogens (Parma et al., 2020; Apper et al., 2016). In addition, a
490 wider range of bacteria supported by a higher diversity may promote a more diverse
491 number of host functions (Solé-Jiménez et al., 2021). However, low level of SPC (SCP10)
492 lead to a lower alpha diversity indexes compared to the control diet which further supports
493 the SCP dose effects on microbial diversity. In agreement with our study, a low dietary
494 inclusion level (equal to 5%) of autolysed yeast tended to reduce gut microbial alpha
495 diversity compared to a vegetable-based control diet in gilthead sea bream (Rimoldi et
496 al., 2020). In addition, the inclusion of brewer's yeast hydrolysate at 0.1 and 0.2% reduced
497 microbial diversity in largemouth bass (*Micropterus salmoides*) (Zhou et al., 2018). The

498 authors postulated a direct effect of specific molecules in the yeast such as nucleotides,
499 mannan oligo saccharide and β -glucan which could inhibit or promote specific bacterial
500 taxa (Zhou et al., 2018). In line with this hypothesis more recently Song et al. (2022)
501 observed in *Litopeneus vannamei* an unexpected decrease of gut microbiota diversity
502 when the guanosine 5'-monophosphate nucleotide was supplemented at 0.1% in diets
503 containing fermented soy in comparison to a non-supplemented diet. Concerning the
504 specific gut microbiota compositional changes, SCP inclusion led to increased *Bacillus*,
505 *Escherichia-shigella* and *Oceanobacillus*. In addition, taxa belonging to *Clostridiaceae* 1
506 family were also responsible for the separation between SCP treatments and control diet.
507 Interestingly, sea bream fed 5% dietary inclusion level of autolysed dried yeast in low
508 FM diet showed an enrichment in *Bacillales* and *Clostridiales* as compared to a control
509 vegetable-based diet (Rimoldi et al., 2020). *Bacillus* is one of the most important
510 beneficial taxa in fish species, which can make a positive contribution to nutrition, to the
511 immune system, and to disease resistance (Busti et al., 2020b; Soltani et al., 2019). In
512 particular *Bacillus spp.* showed growth and feed digestibility improvement mediated by
513 the production of exogenous enzymes (protease, lipase, phytase, chitinase), by the
514 degradation of plant-derived anti-nutritional factors, and by increasing nutrient levels
515 through microbial synthesis of essential bio-molecules (i.e. amino acids, fatty acids, and
516 vitamins). Against pathogens, bacteriocins from *Bacillus spp.* possess a broader spectrum
517 of inhibition that may include Gram-negative and Gram-positive bacteria of genera of
518 *Aeromonas*, *Edwardsiella*, *Streptococcus*, *Pseudomonas*, and *Vibrio*. In addition, oral
519 administration of *Bacillus* strains as probiotics has increased immune parameters (Ringo
520 et al., 2020). This includes lysozyme, phagocytosis, nitric oxide, bactericidal activity,
521 immune genes expression, humoral skin mucus parameters (Soltani et al., 2019) or may

affect the immune system by decreasing inflammation via the up-regulated secretion of anti-inflammatory cytokines (Busti et al., 2020b). Overall these effects of SCP on GM could partially explain the maintenance of the optimal productive results achieved under all SCP dietary inclusion level.

Conclusion

In conclusion, the results of feed intake, growth, feed utilization and gut histology indicate that bacterial SCP from *Corinebacterium glutamicum* can be successfully incorporated up to 20% in practical aquafeed diets to reduce vegetable protein ingredients (total replacement of soy protein concentrate and partial replacement of corn gluten) without any negative effects on growth, protein utilization and gut health during the on-growing phase of gilthead sea bream. The inclusion of SCP at each level tested, determined a shift in the gut microbiota structure promoting taxa such as *Bacillus spp*, which is considered one of the most important beneficial taxa in fish species.

Author's contributions

Conceptualization A.B., L.P., S.F., P.P.G.; Methodology A.M., A.B., L.P., S.F., D.S., M.C., M.M., G.L., P.C., A.D.M., F.D.; Investigation A.M., A.B., L.P., S.F., D.S., M.C., M.M., G.L., P.C., F.D., A.D.M.; Writing-original draft preparation A.M., D.S., L.P.; Writing-review and Editing A.M., A.B., L.P., S.F., D.S., M.C., M.M., G.L., P.C., F.D. All authors have read and agreed to the published version of the manuscript.

Data availability

All data are available in this manuscript

Declaration of Competing Interest

The authors claim that there is no conflict of interest

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References

- Aas, T.S., Grisdale-Helland, B., Terjesen, B.F., Helland, S.J., 2006. Improved growth and nutrient utilisation in Atlantic salmon (*Salmo salar*) fed diets containing a bacterial protein meal. *Aquaculture* 259, 365-376. <https://doi.org/10.1016/j.aquaculture.2006.05.032>.
- Adeoye, A.A., Akegbejo-Samsons, Y., Fawole, F.J., Olatunji, P.O., Muller, N., Wan, A.H.L., Davies, S.J., 2021. From waste to feed: Dietary utilisation of bacterial protein from fermentation of agricultural wastes in African catfish (*Clarias gariepinus*) production and health. *Aquaculture* 531, art. no. 735850 <https://doi.org/10.1016/j.aquaculture.2020.735850>.
- Agboola, J.O., Øverland, M., Skrede, A., Hansen, J.Ø., 2021. Yeast as major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production». *Rev. Aquac.* 13 (2): 949–70. <https://doi.org/10.1111/raq.12507>.

570 Antonopoulou, E., Nikouli, E., Piccolo, G., Gasco, L., Gai, F., Chatzifotis, S., Mente, E.,
 571 Kormas, K.A., 2019. Reshaping gut bacterial communities after dietary *Tenebrio*
 572 *molitor* larvae meal supplementation in three fish species. *Aquaculture* 503, 628-
 573 635.

574 Apper, E., Weissman, D., Respondek, F., Guyonvarch, A., Baron, F., Boisot, P., Rodiles,
 575 A., Merrifield, D.L., 2016. Hydrolysed wheat gluten as part of a diet based on
 576 animal and plant proteins supports good growth performance of Asian seabass
 577 (*Lates calcarifer*), without impairing intestinal morphology or microbiota
 578 *Aquaculture* 453, 40-48.

579 Aruna, T.E., Aworh, O.C., Raji, A.O., Olagunju, A.I., 2017. Protein enrichment of yam
 580 peels by fermentation with *Saccharomyces cerevisiae* (BY4743). *Ann. Agric. Sci.*
 581 62, 33–37. <https://doi.org/10.1016/j.aosas.2017.01.002>.

582 Biswas, A., Takakuwa, F., Yamada, S., Matsuda, A., Saville, R.M., LeBlanc, A.,
 583 Silverman, J.A., Sato, N., Tanaka, H., 2020. Methanotroph (*Methylococcus*
 584 *capsulatus*, Bath) bacteria meal as an alternative protein source for Japanese
 585 yellowtail, *Seriola quinqueradiata* *Aquaculture* 529, art. no. 735700,
 586 <https://doi.org/10.1016/j.aquaculture.2020.735700>.

587 Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification.
 588 *Can. J. Biochem. Physiol.* 37, 911–917. doi:10.1139/o59-099

589 Bolyen E., Rideout J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith G.A.,
 590 Alexander H., Alm E.J., Arumugam M., Asnicar F., et al., 2019. Reproducible,
 591 interactive, scalable and extensible microbiome data science using QIIME 2. *Nat.*
 592 *Biotechnol.* 37 (8), 852–857. doi: 10.1038/s41587-019-0252-6.

593 Bonaldo, A., Roem, A.J., Fagioli, P., Pecchini, A., Cipollini, I., Gatta, P.P., 2008.
594 Influence of dietary levels of soybean meal on the performance and guthistology
595 of gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus*
596 *labrax* L.). *Aquac. Res.* 39, 970–978.

597 Bonvini, E, Bonaldo, A., Mandrioli, L., Sirri, R., Dondi, F., Bianco, C., Fontanillas, R.,
598 Mongile, F., Gatta, P. P., Parma, L., 2018. Effects of feeding low fishmeal diets
599 with increasing soybean meal levels on growth, gut histology and plasma
600 biochemistry of sea bass. *Animal* 12 (5), 923–772.
601 <https://doi.org/10.1017/S1751731117002683>.

602 Busti, S., Rossi, B., Volpe, E., Ciulli, S., Piva, A., D’Amico, F., et al., 2020a. Effects of
603 dietary organic acids and nature identical compounds on growth, immune
604 parameters and gut microbiota of European sea bass. *Sci. Rep.* 10:21321. doi:
605 10.1038/s41598-020-78441-9.

606 Busti, S., Bonaldo, A., Dondi, F., Cavallini, D., Yúfera, M., Gilannejad, N., Moyano, F.,
607 Gatta, P., Parma, L., 2020b. Effects of different feeding frequencies on growth,
608 feed utilisation, digestive enzyme activities and plasma biochemistry of gilthead
609 sea bream (*Sparus aurata*). *Aquaculture* 529, 735616.
610 <https://doi.org/10.1016/j.aquaculture.2020.735616>.

611 Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes,
612 S. P., 2016. DADA2: high-resolution sample inference from Illumina amplicon
613 data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

614 Carvalho, M., Montero, D., Rosenlund, G., Fontanillas, R., Ginés, R., Izquierdo, M.,
615 2020. Effective complete replacement of fish oil by combining poultry and
616 microalgae oils in practical diets for gilthead sea bream (*Sparus aurata*)

617 fingerlings. Aquaculture 529, art. no. 735696,
618 <https://doi.org/10.1016/j.aquaculture.2020.735696>.

619 Chen, Y., Sagada, G., Xu, B., Chao, W., Zou, F., Ng, W.-K., Sun, Y., Wang, L., Zhong,
620 Z., Shao, Q., 2020. Partial replacement of fishmeal with *Clostridium*
621 *autoethanogenum* single-cell protein in the diet for juvenile black sea bream
622 (*Acanthopagrus schlegelii*). Aquaculture Research, 51 (3), 1000-1011.
623 <https://doi.org/10.1111/are.14446>

624 Ciji, A., Akhtar. M. S., 2021. Stress Management in Aquaculture: A Review of Dietary
625 Interventions. Rev. Aquac. raq.12565. <https://doi.org/10.1111/raq.12565>.

626 Culhane, A. C., Thioulouse, J., Perrière, G., and Higgins, D. G., 2005. MADE4: an R
627 package for multivariate analysis of gene expression data. Bioinformatics 21,
628 2789–2790. doi: 10.1093/bioinformatics/bti394.

629 Delamare-Deboutteville, J., Batstone, D.J., Kawasaki, M., Stegman, S., Salini, M.,
630 Tabrett, S., Smullen, R., Barnes, A.C., Hülsen, T., 2019. Mixed culture purple
631 phototrophic bacteria is an effective fishmeal replacement in aquaculture. Water
632 Research X, 4, art. no. 100031 <https://doi.org/10.1016/j.wroa.2019.100031>.

633 Gamboa-Delgado, J., Márquez-Reyes, J.M., 2018. Potential of microbial-derived
634 nutrients for aquaculture development. Rev. Aquac. 10, 224-246.
635 <https://doi.org/10.1111/raq.12157>.

636 Glencross, B. D., Huyben, D., Schrama, J. W., 2020. The Application of Single-Cell
637 Ingredients in Aquaculture Feeds A Review. *Fishes* 5, 22.
638 <https://doi.org/10.3390/fishes5030022>.

639 Guo, J., Qiu, X., Salze, G., Davis, D.A., 2019. Use of high-protein brewer's yeast
640 products in practical diets for the Pacific white shrimp *Litopenaeus vannamei*.
641 Aquac. Nutr., 25 (3), 680-690. <https://doi.org/10.1111/anu.12889>.

642 Hansen, J.Ø., Lagos, L., Lei, P., Reveco-Urzua, F.E., Morales-Lange, B., Hansen, L.D.,
643 Schiavone, M., Mydland, L.T., Arntzen, M.Ø., Mercado, L., Benicio, R.T.,
644 Øverland, M., 2021. Down-stream processing of baker's yeast (*Saccharomyces*
645 *cerevisiae*) Effect on nutrient digestibility and immune response in Atlantic
646 salmon (*Salmo salar*) Aquaculture, 530, art. no. 735707,
647 <https://doi.org/10.1016/j.aquaculture.2020.735707>.

648 Hardy, R.W., Patro, B., Pujol-Baxley, C., Marx, C.J., Feinberg, L., 2018. Partial
649 replacement of soybean meal with *Methylobacterium extorquens* single-cell
650 protein in feeds for rainbow trout (*Oncorhynchus mykiss* Walbaum) Aquac. Res.
651 49 (6), 2218-2224 <https://doi.org/10.1111/are.13678>.

652 Hart, B., Schurr, R., Narendranath, N., Kuehnle, A., Colombo, S. M., 2021. Digestibility
653 of *Schizochytrium* Sp. Whole Cell Biomass by Atlantic Salmon (*Salmo Salar*).
654 Aquaculture 533, 736156. <https://doi.org/10.1016/j.aquaculture.2020.736156>.

655 Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., 2016a. Dietary
656 nucleotide administration influences growth, immune responses and oxidative
657 stress resistance of juvenile red sea bream (*Pagrus major*). Aquaculture 455 41–
658 49.

659 Hossain, M. S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N. M., Ono, S., Fujieda,
660 T., 2016b. Comparison of the effects of inosine and inosine monophosphate on
661 growth, immune response, stress resistance and gut morphology of juvenile red

662 sea bream, *Pagrus major*. *Aquaculture* 458, 64–74.
 663 <https://doi.org/10.1016/j.aquaculture.2016.02.032>
 664 Hossain, M.S., Koshio, S., Kestemont, P., 2020. Recent advances of nucleotide nutrition
 665 research in aquaculture: a review. *Rev. Aquac.* 12 (2), 1028-1053
 666 <https://doi.org/10.1111/raq.12370>.
 667 Jones, S.W., Karpol, A., Friedman, S., Maru, B.T., Tracy, B.P., 2020. Recent advances
 668 in single cell protein use as a feed ingredient in aquaculture. *Curr. Opin.*
 669 *Biotechnol.* 61, 189-197. <https://doi.org/10.1016/j.copbio.2019.12.026>.
 670 Mancera, M., J., Laiz Carrión, R., Del Pilar Martín del Río, M., 2002. Osmoregulatory
 671 action of PRL, GH, and cortisol in the gilthead seabream (*Sparus aurata* L.) (2002)
 672 *Gen. Comp. Endocrinol.* 129 (2), 95-103. [https://doi.org/10.1016/S0016-](https://doi.org/10.1016/S0016-6480(02)00522-1)
 673 [6480\(02\)00522-1](https://doi.org/10.1016/S0016-6480(02)00522-1).
 674 Mateus, A.P., Costa, R., Gisbert, E., Pinto, P.I.S., Andree, K.B., Estévez, A., Power,
 675 D.M., 2017. Thermal imprinting modifies bone homeostasis in cold-challenged
 676 sea bream (*Sparus aurata*). *J. Exp. Biol.* 220 (19), 3442-3454.
 677 <https://doi.org/10.1242/jeb.156174>.
 678 Maulu, S., Liang, H., Ge, X., Yu, H., Huang, D., Ke, J., Ren, M., Mi, H., 2021. Effect of
 679 dietary *Clostridium autoethanogenum* protein on growth, body composition,
 680 plasma parameters and hepatic genes expression related to growth and
 681 AMPK/TOR/PI3K signaling pathway of the genetically improved farmed tilapia
 682 (GIFT: *Oreochromis niloticus*) juveniles. *Anim. Feed Sci. Technol.* 276, art. no.
 683 114914. <https://doi.org/10.1016/j.anifeedsci.2021.114914>.
 684 Messina, M., Bulfon, C., Beraldo, P., Tibaldi, E., Cardinaletti, G., 2019. Intestinal
 685 Morpho-Physiology and Innate Immune Status of European Sea Bass

686 (Dicentrarchus Labrax) in Response to Diets Including a Blend of Two Marine
 687 Microalgae, Tisochrysis Lutea and Tetraselmis Suecica. Aquaculture 500, 660–
 688 69. <https://doi.org/10.1016/j.aquaculture.2018.09.054>.
 689 Naya-Català, F., Wiggers, G.A., Piazzon, M.C., López-Martínez, M.I., Estensoro, I.,
 690 Calduch-Giner, J.A., Martínez-Cuesta, M.C., Requena, T., Sitjà-Bobadilla, A.,
 691 Miguel, M., Pérez-Sánchez, J., 2021a. Modulation of Gilthead Sea Bream Gut
 692 Microbiota by a Bioactive Egg White Hydrolysate: Interactions Between Bacteria
 693 and Host Lipid Metabolism. Front. Mar. Sci., 8, art. no. 698484.
 694 Naya-Català, F., do Vale Pereira, G., Piazzon, M.C., Fernandes, A.M., Calduch-Giner,
 695 J.A., Sitjà-Bobadilla, A., Conceição, L.E.C., Pérez-Sánchez, J., 2021b. Cross-Talk
 696 Between Intestinal Microbiota and Host Gene Expression in Gilthead Sea Bream
 697 (Sparus aurata) Juveniles: Insights in Fish Feeds for Increased Circularity and
 698 Resource Utilization. Front. Physiol, 12, art. no. 748265.
 699 Oliva-Teles, A., Guedes, M.J., Vachot, C., Kaushik, S.J., 2006. The effect of nucleic acids
 700 on growth, ureagenesis and nitrogen excretion of gilthead sea bream Sparus aurata
 701 juveniles. Aquaculture 253 (1-4), 608-617.
 702 <https://doi.org/10.1016/j.aquaculture.2005.09.010>.
 703 Panteli, N., Mastoraki, M., Lazarina, M., Chatzifotis, S., Mente, E., Kormas, K.Ar.,
 704 Antonopoulou, E., 2021. Configuration of gut microbiota structure and potential
 705 functionality in two teleosts under the influence of dietary insect meals.
 706 Microorganisms, 9 (4), art. no. 699.
 707 Parma, L., Candela, M., Soverini, M., Turrone, S., Consolandi, C., Brigidi, P., et al., 2016.
 708 Next-generation sequencing characterization of the gut bacterial community of
 709 gilthead sea bream (Sparus aurata, L.) fed low fishmeal based diets with increasing

soybean meal levels. Anim. Feed Sci. Technol. 222, 204– 216. doi:
10.1016/j.anifeedsci.2016.10.022.

Parma, L., Pelusio, N. F., Gisbert, E., Esteban, M. A., D’Amico, F., Soverini, M.,
Candela, M., 954 Dondi, F., Gatta, P. P., Bonaldo, A., 2020. Effects of rearing
density on growth, digestive 955 conditions, welfare indicators and gut bacterial
community of gilthead sea bream (*Sparus aurata*, 956 L. 1758) fed different
fishmeal and fish oil dietary levels. Aquaculture, 518, 734854. 957
doi:10.1016/j.aquaculture.2019.734854

Pelusio, N. F., Scicchitano, D., Parma, L., Dondi, F., Brini, E., D’Amico, F., Candela, M.,
Yúfera, M., Gilannejad, N., Moyano, F. J., Gatta, P. P., Bonaldo, A., 2021.
Interaction between dietary lipid level and seasonal temperature changes in
gilthead sea bream *Sparus aurata*: effects on growth, fat deposition, plasma
biochemistry, digestive enzyme activity, and gut bacterial community. Front. Mar.
Sci. 8(664701), 1–19. doi:10.3389/fmars.2021.66470.

Peres, H., Santos, S., and Oliva-Teles, A., 2013. Selected plasma biochemistry parameters
in gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 29, 630–636.
doi: 10.1111/j.1439-0426.2012.02049.x.

Quast, C., Klindworth, A., Pruesse, E., Schweer, T., Horn, M., and Glo, F. O., 2013.
Evaluation of general 16S ribosomal RNA gene PCR primers for classical and
next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, 1–11.
doi: 10.1093/nar/gks808.

Rawling, M. D., Pontefract, N., Rodiles, A., Anagnostara, I., Leclercq, E., Schiavone, M.,
Castex, M., Merrifield, D.L. 2019. The Effect of Feeding a Novel Multistrain
Yeast Fraction on European Seabass (*Dicentrarchus Labrax*) Intestinal Health and

734 Growth Performance. J. World Aquacult. Soc. 50 (6): 1108–22.
 735 <https://doi.org/10.1111/jwas.12591>.

736 Rey, A.L., Asín, J., Ruiz Zarzuela, I., Luján, L., Iregui, C.A., de Blas, I., 2020. A proposal
 737 of standardization for histopathological lesions to characterize fish diseases. Rev.
 738 Aquac. 12 (4), 2304-2315rev.

739 Rimoldi, S., Gini, E., Koch, J. F. A., Iannini, F., Brambilla, F., Terova, G., 2020. Effects
 740 of Hydrolyzed Fish Protein and Autolyzed Yeast as Substitutes of Fishmeal in the
 741 Gilthead Sea Bream (*Sparus Aurata*) Diet, on Fish Intestinal Microbiome. BMC
 742 Vet. Res. 16 (1): 118. <https://doi.org/10.1186/s12917-020-02335-1>.

743 Ringø, E., Van Doan, H., Lee, S. H., Soltani, M., Hoseinifar, S. H., Harikrishnan, R., et
 744 al., 2020. Probiotics, lactic acid bacteria and bacilli: interesting supplementation
 745 for aquaculture. J. Appl. Microbiol. 129, 116–136. doi: 10.1111/jam.14628

746 Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F., 2016. VSEARCH: a
 747 versatile open source tool for metagenomics. PeerJ 2016:e2584. doi:
 748 10.7717/peerj.2584

749 Romarheim, O.H., Øverland, M., Mydland, L.T., Skrede, A., Landsverk, T., 2011.
 750 Bacteria grown on natural gas prevent soybean meal-induced enteritis in atlantic
 751 salmon. J. Nutr. 141, 124-130. <https://doi.org/10.3945/jn.110.128900>.

752 Sharif, M., Zafar, M.H., Aqib, A.I., Saeed, M., Farag, M.R., Alagawany, M., 2021. Single
 753 cell protein: Sources, mechanism of production, nutritional value and its uses in
 754 aquaculture nutrition. Aquaculture, 531, art. no. 735885,
 755 <https://doi.org/10.1016/j.aquaculture.2020.735885>.

756 Smáráson, B.Ö., Alriksson, B., Jóhannsson, R., 2019. Safe and sustainable protein
 757 sources from the forest industry – The case of fish feed. *Trends Food Sci. Technol.*
 758 84, 12-14. <https://doi.org/10.1016/j.tifs.2018.03.005>.
 759 Solé-Jiménez, P., Naya-Català, F., Piazzon, M.C., Estensoro, I., Caldach-Giner, J.À.,
 760 Sitjà-Bobadilla, A., Van Mullem, D., Pérez-Sánchez, J., 2021. Reshaping of Gut
 761 Microbiota in Gilthead Sea Bream Fed Microbial and Processed Animal Proteins
 762 as the Main Dietary Protein Source. *Front. Mar. Sci*, 8, art. no. 705041
 763 Soltani, M., Ghosh, K., Hoseinifar, S.H., Kumar, V., Lymbery, A.J., Roy, S., Ringø, E.,
 764 2019. Genus bacillus, promising probiotics in aquaculture: Aquatic animal origin,
 765 bio-active components, bioremediation and efficacy in fish and shellfish. *Rev.*
 766 *Fish. Sci. Aquac.*, 27 (3), 331-379.
 767 Song, X., Ye, H., Jin, F., Li, H., Kim, Y.-S., Xiao, J., Guo, Z., 2022. Effects of fermented
 768 soybean meal and guanosine 5'-monophosphate on growth, intestinal health and
 769 anti-stress capability of *Penaeus vannamei* in low fish meal diet. *Aquaculture* 548,
 770 art. no. 737591.
 771 Woodgate, S.L., Wan, A.H.L., Hartnett, F., Wilkinson, R.G., Davies, S.J., 2022. The
 772 utilisation of European processed animal proteins as safe, sustainable and circular
 773 ingredients for global aquafeeds. *Rev. Aquac.*
 774 Zepka, L.Q., Jacob-Lopes, E., Goldbeck, R., Souza-Soares, L.A., Queiroz, M.I., 2010.
 775 Nutritional evaluation of single-cell protein produced by *Aphanothece*
 776 *microscopica* Nägeli. *Bioresour. Technol.* 101 (18), 7118-7122.
 777 <https://doi.org/10.1016/j.biortech.2010.04.001>.

778 Zhang, F., Man, Y.B., Mo, W.Y., Wong, M.H., 2020. Application of Spirulina in
 779 aquaculture: a review on wastewater treatment and fish growth. Rev. Aquac. 12
 780 (2), 582-599. <https://doi.org/10.1111/raq.12341>.
 781 Zhou, M., Liang, R., Mo, J., Yang, S., Gu, N., Wu, Z., Babu, V S., Li, J., Yunmao Huang,
 782 Y., Lin, L., 2018. Effects of brewer's yeast hydrolysate on the growth performance
 783 and the intestinal bacterial diversity of largemouth bass (*Micropterus salmoides*),
 784 Aquaculture, 484, 139-144
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Table 1. Ingredients and proximate composition of the experimental diets

	CTRL	SCP 10	SCP 15	SCP 20
Ingredients, % of the diet				
Fishmeal Super Prime (Peruvian)	18.0	18.0	18.0	18.0
Soy protein concentrate	17.0	8.00	4.00	0.00
Corn gluten	16.8	11.0	8.00	5.50
Soybean meal 48	14.2	12.8	12.4	12.4
Wheat meal	7.60	8.10	8.40	9.00
Fish oil	4.00	4.00	4.00	4.00
Salmon oil	12.0	12.0	12.0	12.0
*Vit & Min Premix INVIVO 1%	1.00	1.00	1.00	1.00
MCP	0.60	0.40	0.30	0.30
L-Lysine	0.10	0.27	0.40	0.50
DL-Methionine	0.04	0.09	0.11	0.11
L-Taurine	0.05	0.06	0.06	0.06
Sunflower meal concentrate	8.61	14.28	16.33	17.13
#NT 70 Gordini	-	10.0	15.0	20.0
Yttrium oxide	0.01	0.01	0.01	0.01
Proximate composition, % on a wet weight basis				
Moisture	6.55	6.78	6.85	6.91
Protein	44.41	44.13	44.44	44.59
Lipid	19.01	18.83	19.04	18.73
Ash	7.12	7.12	7.20	7.33
Gross energy MJ kg ⁻¹	22.41	22.42	22.42	22.42

*Vitamins and mineral premix (IU or mg kg⁻¹ diet; Invivo NSA, Portugal); DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 16,650 IU; DL-cholecalciferol, 2000 IU; thiamine, 25 mg; riboflavin, 25 mg; pyridoxine, 25 mg; cyanocobalamin, 0.1 mg; niacin, 150 mg; folic acid, 15 mg; L-ascorbic acid monophosphate, 750 mg; inositol, 500 mg; biotin, 0.75 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg; copper sulphate heptahydrate, 25 mg; ferric sulphate monohydrate, 100 mg; potassium iodide, 2 mg; manganese sulphate monohydrate, 100 mg; sodium selenite, 0.05 mg; zinc sulphate monohydrate, 200 mg.

Single cell protein, SCP from *Corinebacterium glutamicum*

Table 2. Amino acid composition, g/100g of the bacterial single cell protein (NT 70 Gordini)

Aspartic acid (including Asparagina)	5.48
Glutamic acid (including Glutamine)	7.50
Hydroxyproline	<0.1
Serine	2.26
Glycine	4.15
Histidine	1.18
Arginine	3.36
Threonine	12.6
Alanine	4.16
Proline	2.11
Tyrosine	2.20
Valine	3.26
Methionine	1.36
Isoleucine	2.85
Leucine	4.75
Phenylalanine	2.54
Lysine	3.05
Tryptophane	0.915
Cysteine	0.526

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Table 3. Growth performance measured in gilthead seabream fed increasing dietary single cell protein levels.

	CTRL	SCP10	SCP15	SCP20	<i>P</i> - value
IBW	75.1 ± 2.67	75.5 ± 0.95	75.0 ± 0.56	75.3 ± 2.02	0.98
FBW	216.3 ± 11.41	216.6 ± 5.95	212.2 ± 2.48	214.6 ± 3.69	0.831
WG	141.2 ± 8.74	141.2 ± 5.95	136.9 ± 2.69	139.3 ± 4.46	0.776
SGR	0.98 ± 0.02	0.98 ± 0.01	0.96 ± 0.01	0.97 ± 0.03	0.653
FCR	1.29 ± 0.04	1.28 ± 0.05	1.35 ± 0.01	1.35 ± 0.05	0.122
FI	1.16±0.03	1.16±0.04	1.20±0.02	1.22±0.03	0.124
Survival	97.80 ± 2.2	96.30 ± 1.3	98.5 ± 2.6	97.80 ± 0.00	0.531
ECR	1.63 ± 0.06	1.53 ± 0.06	1.58 ± 0.02	1.54 ± 0.05	0.136

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

IBW= Initial body weight (g).

FBW = Final body weight (g).

WG = Weight gain (g).

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

FCR = Feed conversion rate = feed intake / weight gain.

FI = Feed intake (g kg ABW⁻¹ day⁻¹) = ((100*total ingestion)/(ABW))/(days)).

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

Survival = Survival (%).

Economic conversion ratio (€/kg fish⁻¹) = FCR * feed cost

Table 4. Body composition, nutritional indices, somatic indices and apparent digestibility measured in gilthead seabream fed increasing dietary single cell protein levels.

	CTRL	SCP10	SCP15	SCP20	<i>P-value</i>
<i>Whole body composition, %</i>					
Moisture	62.0±0.87	61.5±1.03	62.1±0.88	62.2±0.51	0.719
Protein	17.8±0.08 ^b	17.3±0.08 ^{ab}	17.2±0.03 ^a	17.5±0.06 ^{ab}	0.025
Lipid	16.9±1.29	17.0±1.17	16.9±0.80	16.7±0.46	0.990
Ash	3.4±0.16	3.4±0.22	3.5±0.19	3.1±0.19	0.257
<i>Nutritional indices, %</i>					
PER	1.52 ± 0.01	1.54 ± 0.02	1.52 ± 0.01	1.51 ± 0.01	0.178
GPE	28.0 ± 1.30 ^b	26.7 ± 0.22 ^{ab}	25.7 ± 0.72 ^a	26.3 ± 0.45 ^{ab}	0.015
<i>Apparent digestibility, %</i>					
Dry matter digestibility	92.9 ± 3.1	94.1 ± 0.9	93.4 ± 1.4	94.4 ± 1.1	0.761
Protein	95.5 ± 1.57	96.0 ± 0.16	95.5 ± 1.42	96.5 ± 0.90	0.307
<i>Somatic indices</i>					
CF	1.84 ± 0.17 ^{ab}	1.85 ± 0.15 ^b	1.78 ± 0.16 ^{ab}	1.73 ± 0.18 ^a	0.018
VSI	6.17 ± 1.02	5.65 ± 1.32	5.98 ± 0.77	5.93 ± 1.30	0.628
HSI	1.22 ± 0.22	1.35 ± 0.24	1.36 ± 0.25	1.31 ± 0.26	0.403

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

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

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

HSI = Hepatosomatic index (%) = $100 * (\text{liver weight} / \text{FBW})$.

VSI = Viscerosomatic index (%) = $100 * (\text{viscera weight} / \text{FBW})$.

SD = Standard deviation.

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Table 5. Absorbed surface of the anterior and middle intestine evaluated in gilthead seabream by means of binarization method

	CTRL	SCP10	SCP15	SCP20	<i>P-value</i>
Anterior absorbent surface	3.68 ± 2.0	4.15 ± 1.5	3.76 ± 1.0	3.71 ± 1.2	> 0.05
Middle absorbent surface	1.38 ± 0.8	1.22 ± 0.4	1.33 ± 0.6	1.60 ± 0.8	> 0.05
Data are given as the tanks mean (n=12 ± SD)					

Table 6. Plasma biochemistry values measured in gilthead seabream fed increasing dietary single cell protein levels.

	CTRL	SCP10	SCP15	SCP20	<i>P</i> - value
GLU	160.4 ± 20.03	160.0 ± 16.12	147.3 ± 11.91	147.6 ± 14.41	0.5669
Urea	12.4 ± 0.99 ^a	13.3 ± 1.81 ^a	13.7 ± 0.73 ^a	16.5 ± 1.41 ^b	0.0006
CREA	0.25 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.27 ± 0.04	0.3445
Uric Ac	0.21 ± 0.11	0.15 ± 0.03	0.18 ± 0.13	0.24 ± 0.12	0.5495
Tot Bil	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.06 ± 0.01	0.0625
CHOL	264.0 ± 11.8	318.1 ± 17.6	301.8 ± 21.0	321.0 ± 33.4	0.0524
TRIG	555.4 ± 58.1 ^a	756.7 ± 30.6 ^{ab}	775.6 ± 198.0 ^b	754.5 ± 88.2 ^{ab}	0.0172
HDL	92.5 ± 4.14 ^a	109.8 ± 5.23 ^{ab}	106.8 ± 10.6 ^{ab}	114.6 ± 17.9 ^b	0.0337
TP	3.45 ± 0.21	3.60 ± 0.38	3.61 ± 0.28	3.69 ± 0.35	0.2625
ALB	0.87 ± 0.09	0.87 ± 0.13	0.87 ± 0.09	0.89 ± 0.09	0.9538
AST	59.6 ± 8.49	58.8 ± 15.3	66.7 ± 46.3	62.0 ± 12.8	0.9757
ALT	14.6 ± 0.97	16.3 ± 4.11	31.3 ± 36.36	15.7 ± 2.80	0.4859
ALP	230.1 ± 71.3	253.7 ± 40.5	234.3 ± 28.3	273.6 ± 53.0	0.7484
CK	3156 ± 3991	1119 ± 451	1313 ± 1107	2065 ± 1227	0.4770
LDH	504.0 ± 248.6	289.9 ± 188.5	301.3 ± 236.5	458.2 ± 289.1	0.5189
LACT	31.6 ± 5.38	26.2 ± 3.96	30.8 ± 9.52	23.2 ± 6.28	0.1364
Ca ⁺²	12.1 ± 0.50 ^a	12.3 ± 0.76 ^{ab}	12.6 ± 0.30 ^{ab}	12.8 ± 0.63 ^b	0.0492
P	10.9 ± 0.83 ^a	12.6 ± 1.14 ^b	11.9 ± 0.40 ^{ab}	11.9 ± 0.85 ^{ab}	0.0025
K ⁺	5.90 ± 1.18	6.31 ± 1.17	6.34 ± 0.22	6.16 ± 1.37	0.5291
Na ⁺	181.3 ± 3.58	176.8 ± 2.27	178.9 ± 5.51	179.7 ± 0.98	0.1605
Fe	80.0 ± 8.41	75.9 ± 13.1	77.3 ± 8.88	90.5 ± 17.0	0.2830
Cl	153.1 ± 1.83	148.7 ± 1.04	150.5 ± 4.37	151.3 ± 0.42	0.0637
Mg	2.94 ± 0.25	3.01 ± 0.11	3.08 ± 0.12	3.02 ± 0.09	0.8412
ALB/GLO	0.34 ± 0.02	0.32 ± 0.02	0.32 ± 0.01	0.32 ± 0.01	0.1310
CaxP	132.2 ± 14.11 ^a	155.8 ± 23.60 ^b	149.6 ± 8.27 ^{ab}	154.0 ± 18.1 ^{ab}	0.0113
Na/K	31.7 ± 5.90	29.0 ± 5.47	28.7 ± 0.23	30.4 ± 6.95	0.3155
Cur Ca ⁺²	14.8 ± 0.42 ^a	15.0 ± 0.66 ^{ab}	15.2 ± 0.26 ^{ab}	15.4 ± 0.55 ^b	0.0281

Data are given as the mean (n=15 diet⁻¹) ± SD. Different letters indicate significant difference (One-way ANOVA $P \leq 0.05$) between treatments.

GLU, glucose , (mg dL⁻¹) ; Urea , (mg dL⁻¹) ; CREA, creatinine , (mg dL⁻¹) ; Uric Ac, uric acid , (mg dL⁻¹) ; Tot Bil, total bilirubin , (mg dL⁻¹) ; CHOL, cholesterol , (mg dL⁻¹) ; TRIG, triglycerides , (mg dL⁻¹) ; HDL, high density lipoprotein; TP, total protein , (mg dL⁻¹) ; Alb, albumin , (g dL⁻¹) ; Ast, aspartate aminotransferase , (U L⁻¹) ; Alt, alanine aminotransferase (U L⁻¹) ; Alp, alkaline phosphatase , (U L⁻¹) ; CK, creatine kinase , (U L⁻¹) ; LDH, lactate dehydrogenase , (U L⁻¹) ; LAC, lactate (mmol L⁻¹) ; Ca⁺² , calcium , (mg dL⁻¹) ; P, inorganic phosphorus , (mg dL⁻¹) ; K⁺, potassium , (mEq L⁻¹) ; Na⁺, sodium , (mEq L⁻¹) ; Fe, iron , (µg dL⁻¹) ; Cl, chloride , (mEq L⁻¹) ; Mg, magnesium , (mg dL⁻¹) ; ALB/GLO, albumin/globulin; CaxP, calcium*phosphorus ; Na/K, sodium/potassium ; Cur Ca²⁺, current calcium (mg dL⁻¹) ; SD, standard deviation.

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Table 7. Relative abundance (%) \pm SD of the most abundant phyla and families (all belonging to Firmicutes phylum) in the 4 experimental diet.

	CTRL	SCP10	SCP15	SCP20
Phylum				
Firmicutes	90.3 \pm 6.8	90.0 \pm 4.9	71.7 \pm 13.1	60.4 \pm 17.3
Family				
<i>Staphylococcaceae</i>	32.1 \pm 13.5	46.4 \pm 9.8	37.4 \pm 8.5	19.6 \pm 7.7
<i>Bacillaceae</i>	1.5 \pm 1.2	20.0 \pm 7.4	14.1 \pm 5.1	8.3 \pm 3.6
<i>Lactobacillaceae</i>	8.2 \pm 8.3	10.2 \pm 9.3	7.2 \pm 6.1	7.3 \pm 5.5
<i>Leuconostocaceae</i>	32.4 \pm 18.1	1.3 \pm 1.6	1.4 \pm 1.3	1.1 \pm 1.3

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826 **Figure 1**

827 A-D Process of image binarization. A) Original acquired image with 10x. B) Specific threshold to highlight
828 the absorbent surface of the intestinal tract. C) Region of interest (ROI) used to measure number of binary
829 objects inside each ROI, is indicated by arrow. D) Binary area, sum of areas of all binary objects. It is
830 shown in square pixels and it's indicated in green. In these anterior and middle gut samples it corresponds
831 to the absorbent surface of the intestinal tract.

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833 **Figure 2**

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835 Beta diversity and alpha diversity of gut microbiota of gilthead seabream fed with increasing dietary single
836 cell protein levels (SCP). (A) Principal Coordinates Analysis (PCoA) based on unweighted UniFrac
837 distances between gut microbiota structure of animals fed with CTRL diet, SCP10 diet, SCP15 diet and
838 SCP20 diet. Samples are significantly separated (permutation test with pseudo-F ratios Adonis; $p = 0.001$).
839 Black arrows are obtained by fitting the family relative abundance values for each sample within the
840 ordination space (function envfit of the “vegan” R package, with a p -value < 0.001). (B) Boxplots show
841 alpha diversity values, measured by Faith's Phylogenetic Diversity (PD_whole_tree), Chao1 index, and
842 amplicon sequence variants (observed_ASVs). All metrics showed a significant variation (Kruskal–Wallis
843 test $p < 0.01$) of alpha diversity among dietary groups. More specifically, SCP10 and SCP15 groups showed
844 a significant reduction of the internal ecosystem diversity compared to CTRL group, while the SCP20 group
845 showed a significant opposite variation compared to control group (Wilcoxon rank-sum test, $p < 0.05$).
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847 **Figure 3**

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849 Microbiota composition (%) of distal gut content of gilthead sea bream fed with increasing dietary single
850 cell protein levels (SCP). Bar plot summarizing the microbiota composition at phylum (A) and family level
851 (B) of fish intestinal content. Only phyla with a relative abundance $\geq 1.0\%$ in at least 2 samples, and families
852 with relative abundance $\geq 1.0\%$ in at least 2 samples are represented.
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854 **Figure 4**

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856 Taxonomic composition (%) of bacterial communities of distal gut content of gilthead sea bream fed with
857 increasing dietary single cell protein levels (SCP). Distributions of relative abundance of genera that
858 showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** $p \leq$
859 0.001 ; ** $p \leq 0.01$; * $p \leq 0.05$). Only genera with a mean relative abundance $\geq 1.0\%$ in at least 2 samples
860 were represented. The central box of each dataset represents the distance between the 25th and the 75th
861 percentiles. The median between them is marked with a black line.
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Figure 1

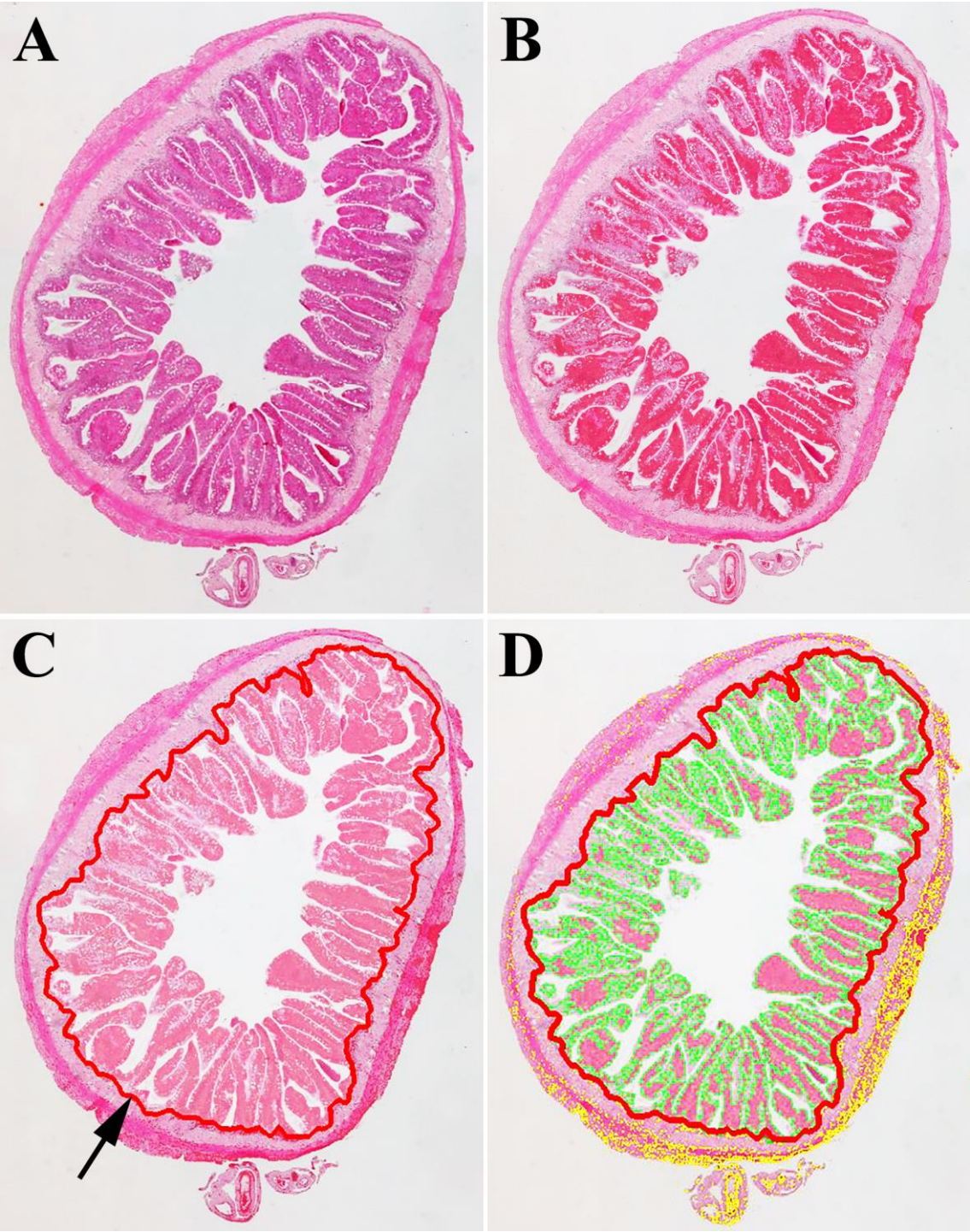
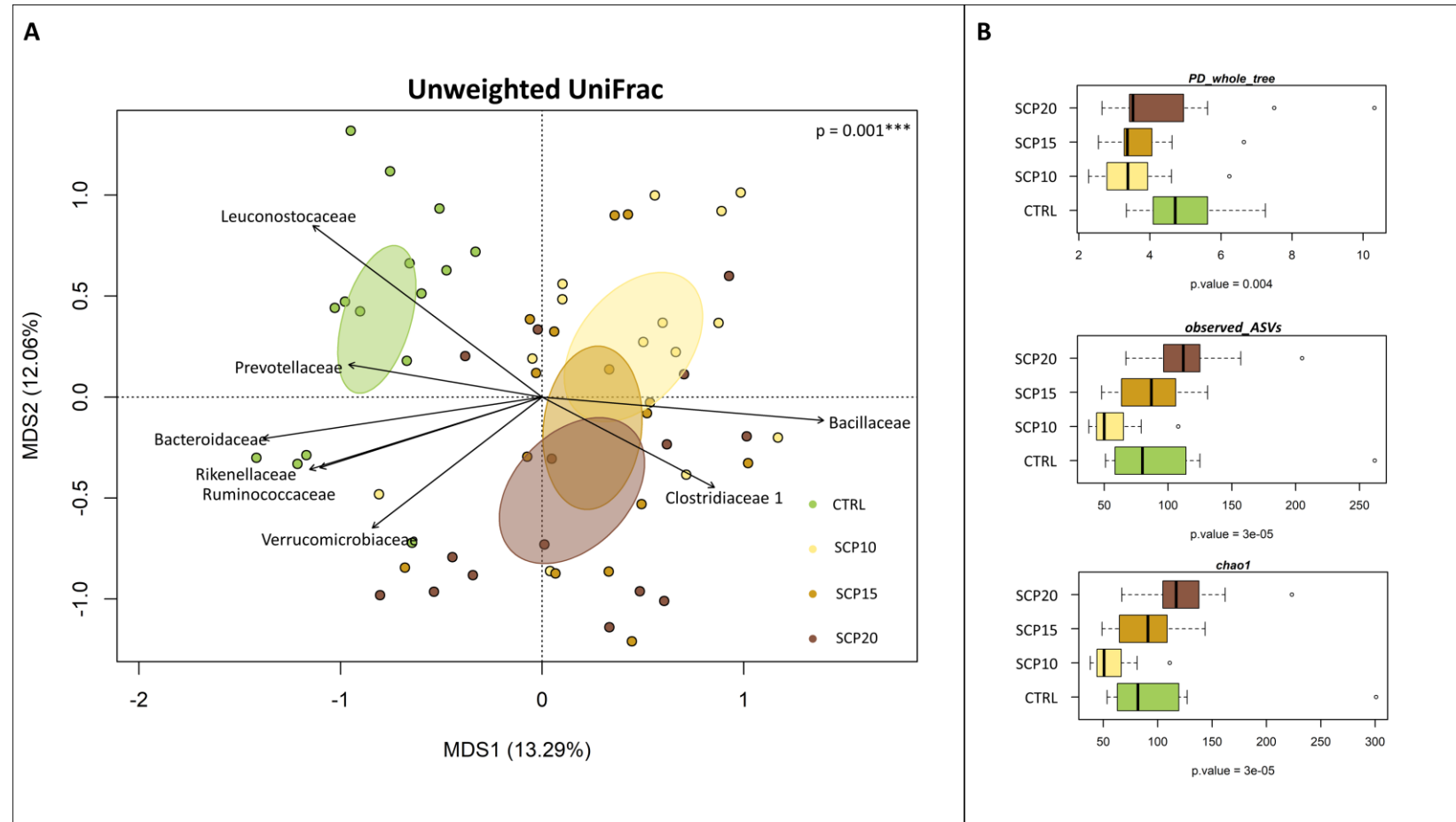
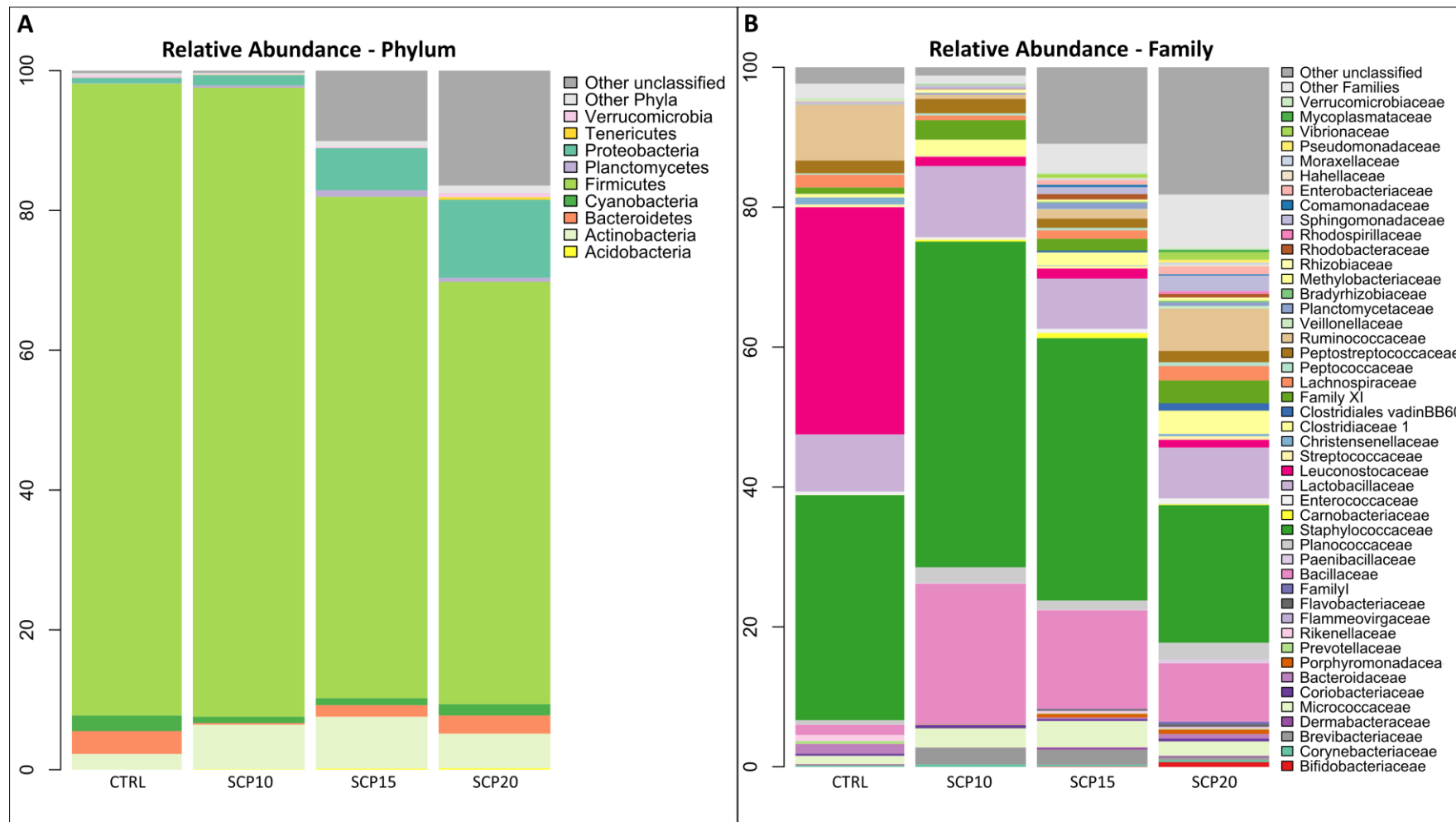


Figure 2

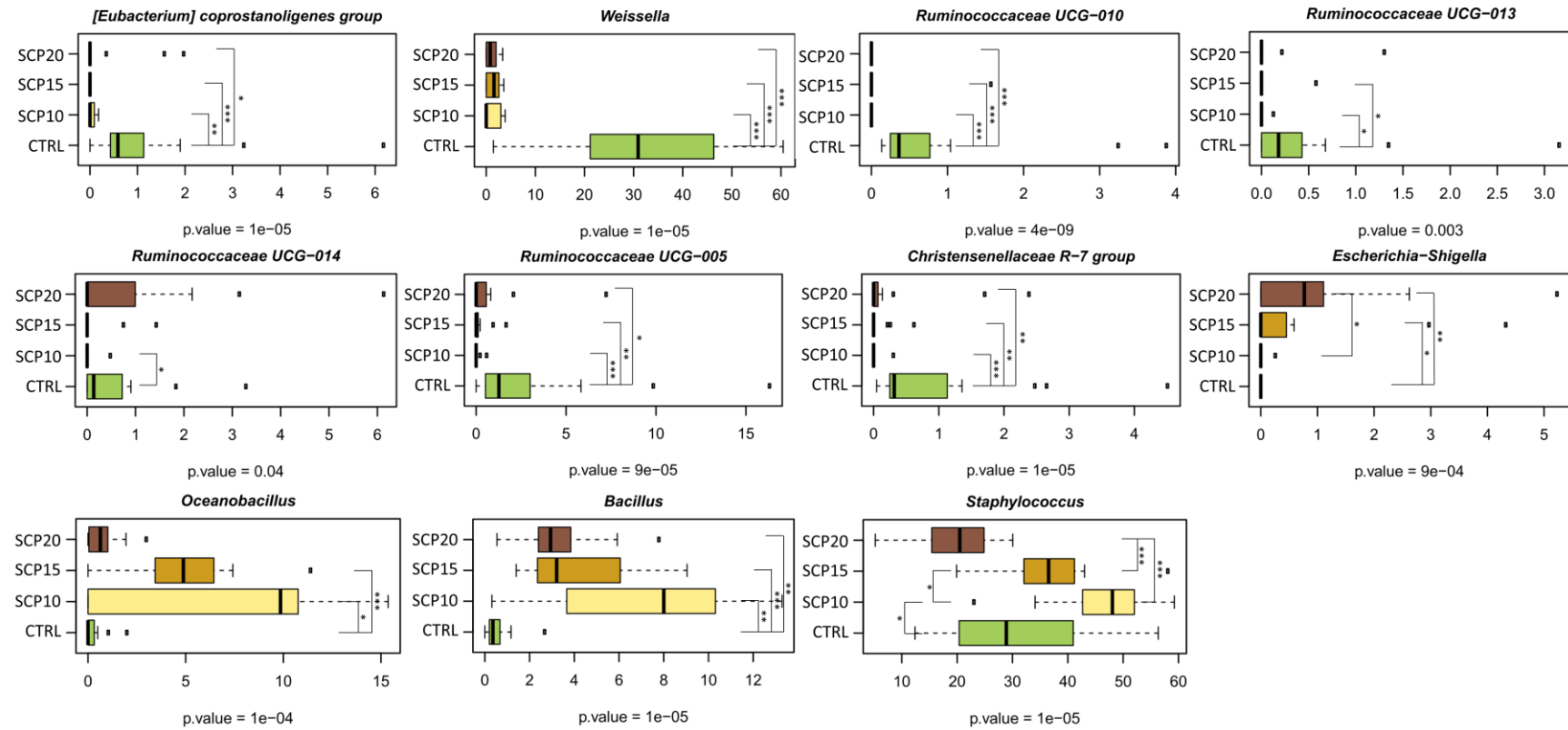


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900 **Figure 4**



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