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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 ± 1.0 mg L^{-1}; Temperature 23 ± 1.0 °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 x *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: ADC = 100*(1−(dietary Y2O2 level/ faecalY2O2 level))*((faecal nutrient/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 (Ca+2), 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 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Economic conversion ratio (ECR) (€/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 ± 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 ± 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

330 Plasma parameters are shown in Table 6. Urea was higher in SCP20 compared to the
331 other groups. TRIG were higher in SCP15 compared to CTRL. HDL was higher in SCP20
332 compared to CTRL. Ca^{+2} and Cur Ca^{+2} were significantly higher in the SCP20 diet than
333 in the CTRL diet, while P and CaxP were higher in SCP10 compared to CTRL. No
334 significant differences among treatments were detected for GLU, CREA, Uric Ac, Tot
335 Bil, CHOL, TP, ALB, AST, ALT, ALP, CK, LDH, LACT, K^{+}, Na^{+}, Fe, Cl, Mg,
336 ALB/GLO, Na/K.
337
338 *3.4 Faecal bacterial community profiles related to dietary groups*
339
340 The 16S rRNA gene sequencing was performed on a total of 60 distal intestine content
341 samples, yielding 401'841 high-quality reads (mean ± SD, 6'182 ± 2'574) and clustered
342 into a total of 6'252 ASVs. To assess whether the treatments with increasing SCP could
343 exert an effect on the gut bacteria community during the growth process of gilthead
344 seabream, the gut microbiota (GM) was analysed for each dietary group at the end of the
345 trial. The GM variations between samples (beta-diversity) were assessed by the Principal
346 Coordinates Analysis (PCoA) based on Unweighted UniFrac distances, with the taxa
347 most explaining sample segregation being superimposed on the bidimensional space. In
348 addition, the gut microbial community internal diversity was represented with three
349 different metrics for each dietary group: PD_whole_tree, Chao1 index, and
350 observed_ASVs. According to the findings (Figure 2A), all SCP groups showed a
351 significant variation compared to the control group, in terms of overall GM composition
352 ("pairwise Adonis permutation test", $p < 0.01$). Focusing on dietary group, a significant
353 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

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

428 Most of the plasma biochemistry results, such as TP, ALB, Glu, TC, and ALT, did not
429 show any statistical differences, demonstrating that SCP could guarantee optimal
430 nutrition and general health status (including liver health status as indicated by AST and
431 ALT) in agreement with previous plasma values on this species and with the results
432 obtained in Nile Tilapia fed bacterial SCP (Parma et al., 2020; Maulu et al., 2021).
433 However, Ca^{+2} and $CurCa^{+2}$ values were higher in SCP20 and, CaxP was higher in SCP10
434 compared to control diet. Differences when compared to the standard calcium values
435 could represent a stress indicator, causing imbalance plasma ion level and increasing
436 plasma osmolality (Mancera et al., 2002; Mateus et al., 2017). Despite this, calcium
437 values shown in this work, are in line with previous works of the same species and within
438 values of healthy fish (Peres et al., 2013; Pelusio et al., 2021). It is also worth highlighting
439 the higher value of plasmatic urea in SCP20. In agreement with Oliva-Teles et al. (2006)
440 high levels of SCP can lead to high levels of non-protein nitrogen content, such as nucleic
441 acid, mostly represented by RNA. Ammonia is the final product of pyrimidines
442 catabolism, which are one of the main components of RNA molecules. Even the higher
443 values of inorganic phosphorus detecting in SCP10 could be related to the degradation of
444 RNA molecules, even if this trend was not observed at the higher inclusion level (SCP15,
445 SCP20) tested. Plasma triglyceride and HDL were higher in the fish fed SCP15 and
446 SCP20 compared with the control diet, respectively. According to Maulu et al. (2021) the
447 inclusion of 200g kg of *Clostridium autoethanogenum* bacterial SCP increased plasma
448 triglycerides and cholesterol in Nile Tilapia. Although it is not clear how SCPs could
449 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;

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

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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	*P - 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

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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	*P-value*
Whole body composition, %					
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*
Nutritional indices, %					
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***
Apparent digestibility, %					
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
Somatic indices					
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*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.

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

VSI = Viscerosomatic index (%) = 100*(viscera weight/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	*P-value*
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	*P - 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 (%) ± 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 ± 6.8	90.0 ± 4.9	71.7 ± 13.1	60.4 ± 17.3
Family				
Staphylococcaceae	32.1 ± 13.5	46.4 ± 9.8	37.4 ± 8.5	19.6 ± 7.7
Bacillaceae	1.5 ± 1.2	20.0 ± 7.4	14.1 ± 5.1	8.3 ± 3.6
Lactobacillaceae	8.2 ± 8.3	10.2 ± 9.3	7.2 ± 6.1	7.3 ± 5.5
Leuconostocaceae	32.4 ± 18.1	1.3 ± 1.6	1.4 ± 1.3	1.1 ± 1.3

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Figure 1

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

Figure 2

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

Figure 3

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

Figure 4

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

Figure 1

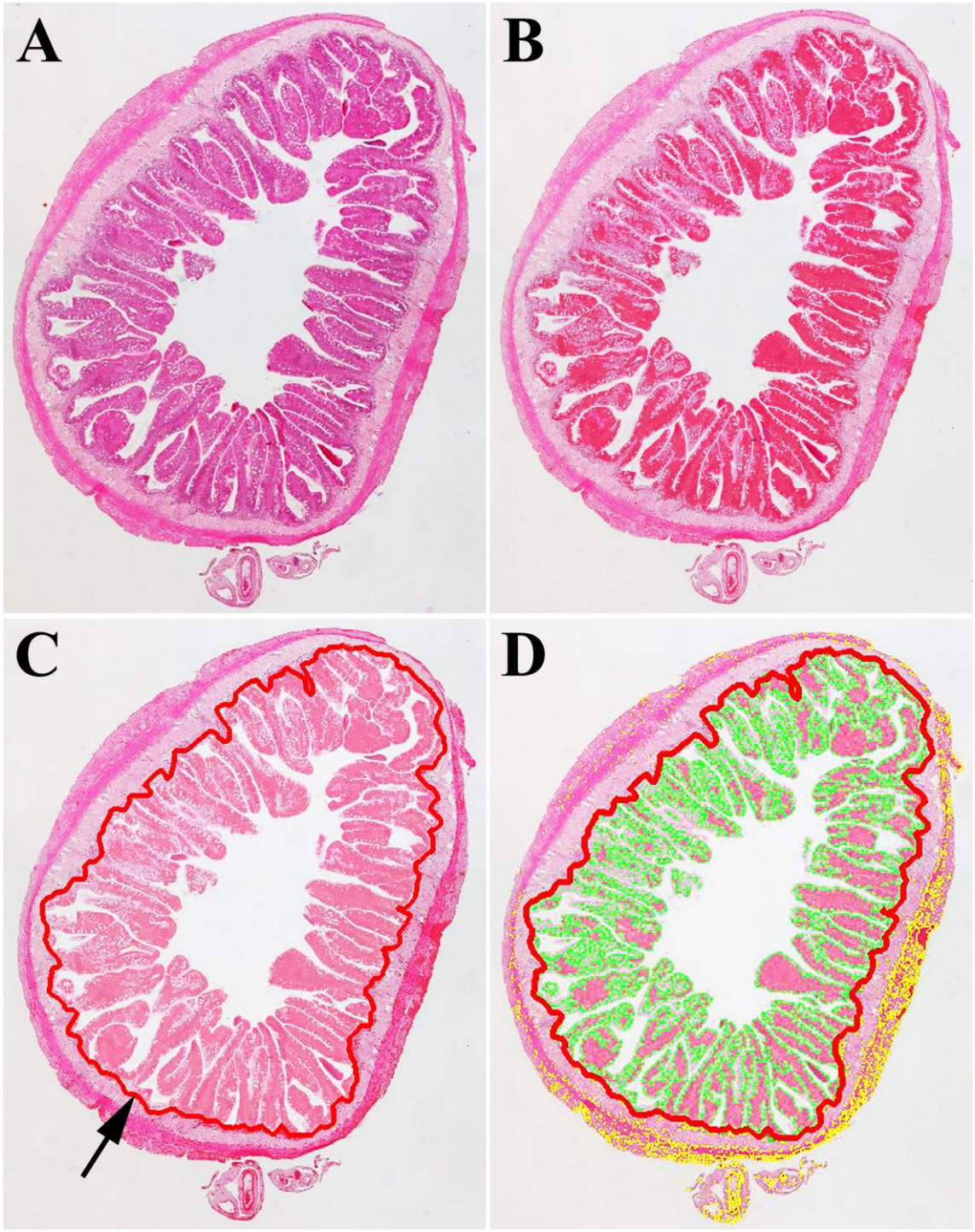


Figure 2

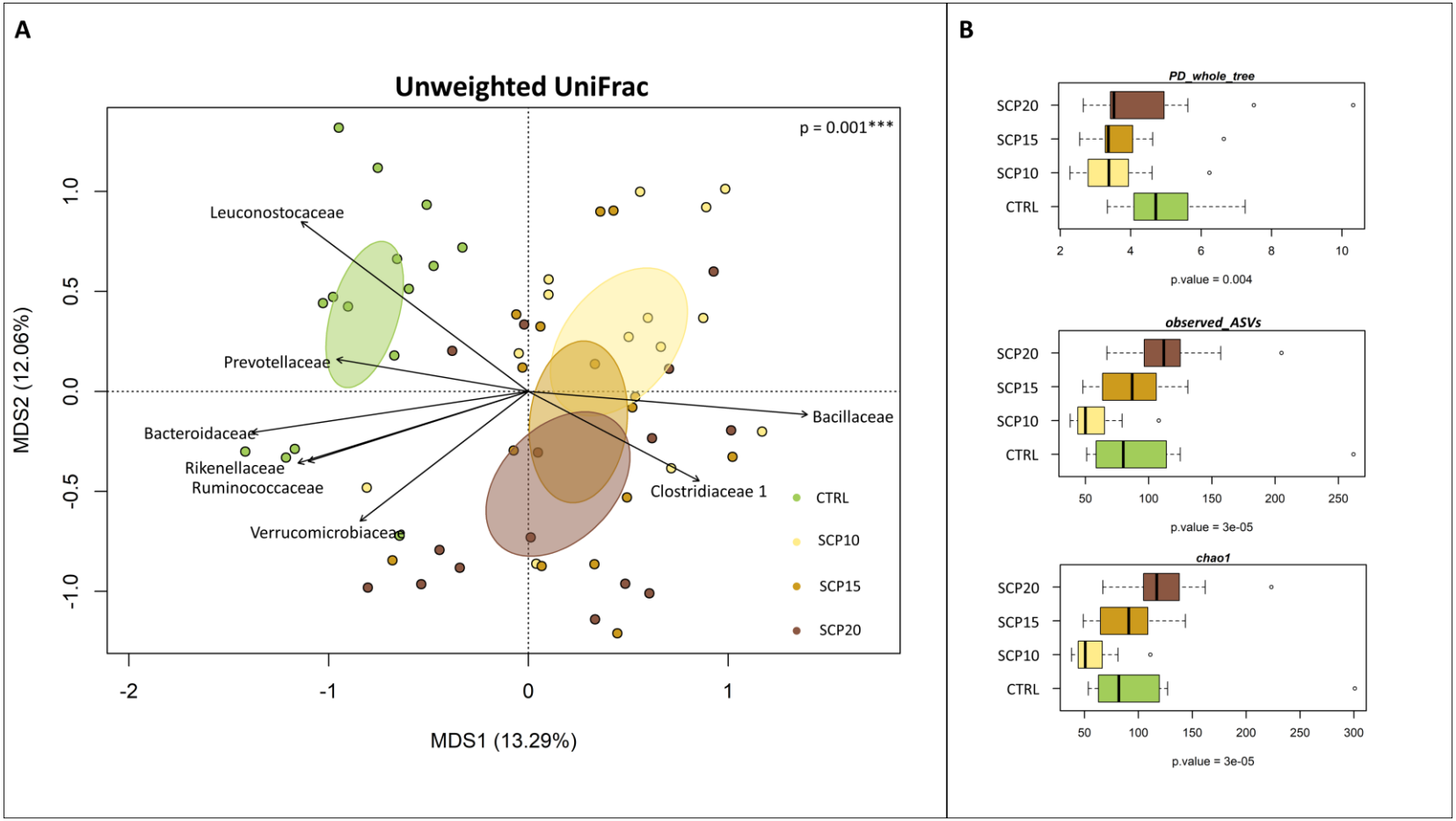

A
Unweighted UniFrac
p = 0.001***
Leuconostocaceae
Prevotellaceae
Bacteroidaceae
Rikenellaceae
Ruminococcaceae
Verrucomicrobiaceae
Bacillaceae
Clostridiaceae 1
CTRL
SCP10
SCP15
SCP20
MDS2 (12.06%)
MDS1 (13.29%)
B
PD_whole_tree
SCP20
SCP15
SCP10
CTRL
p.value = 0.004
observed_ASVs
p.value = 3e-05
chao1
p.value = 3e-05

Figure 3

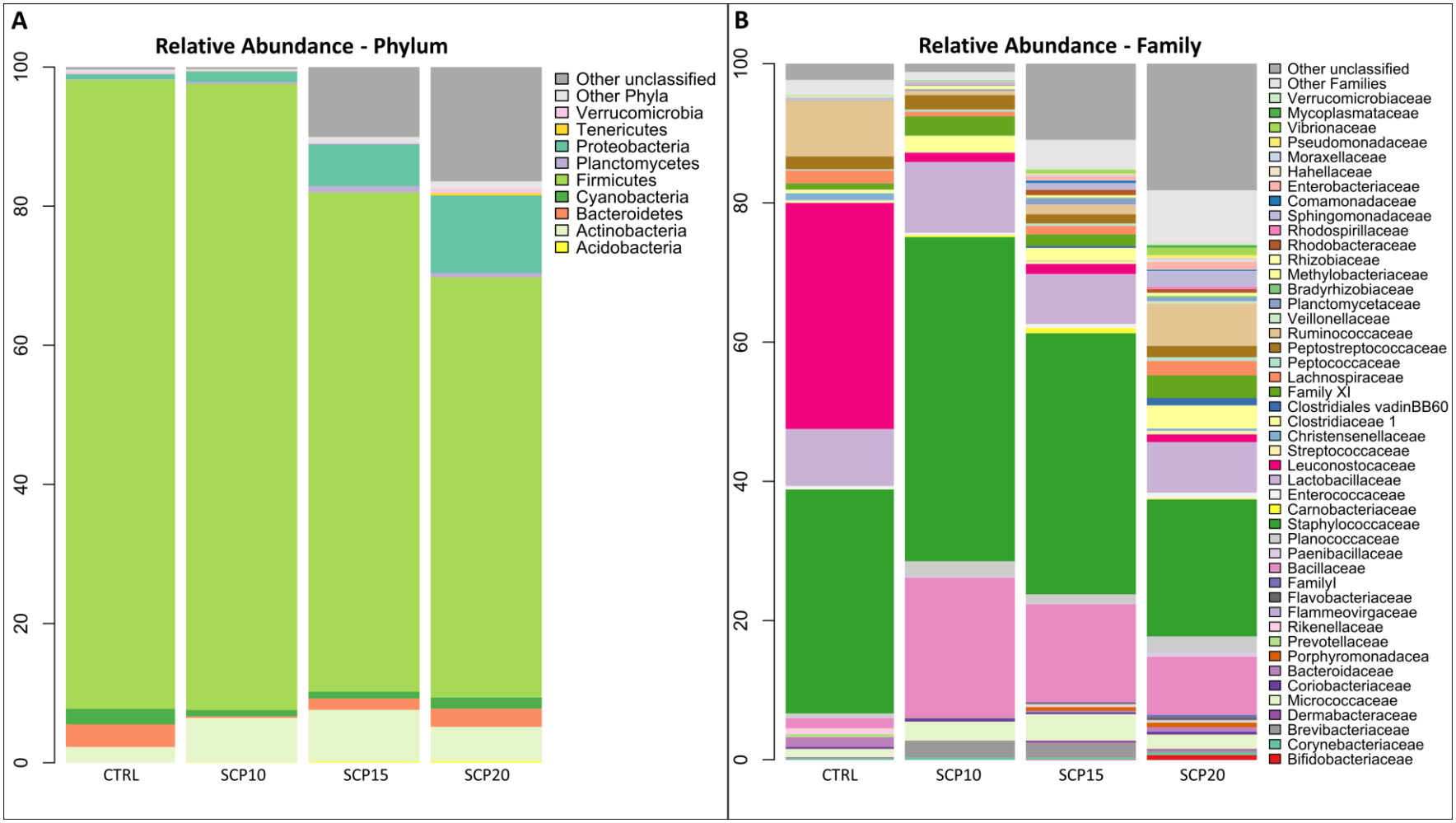
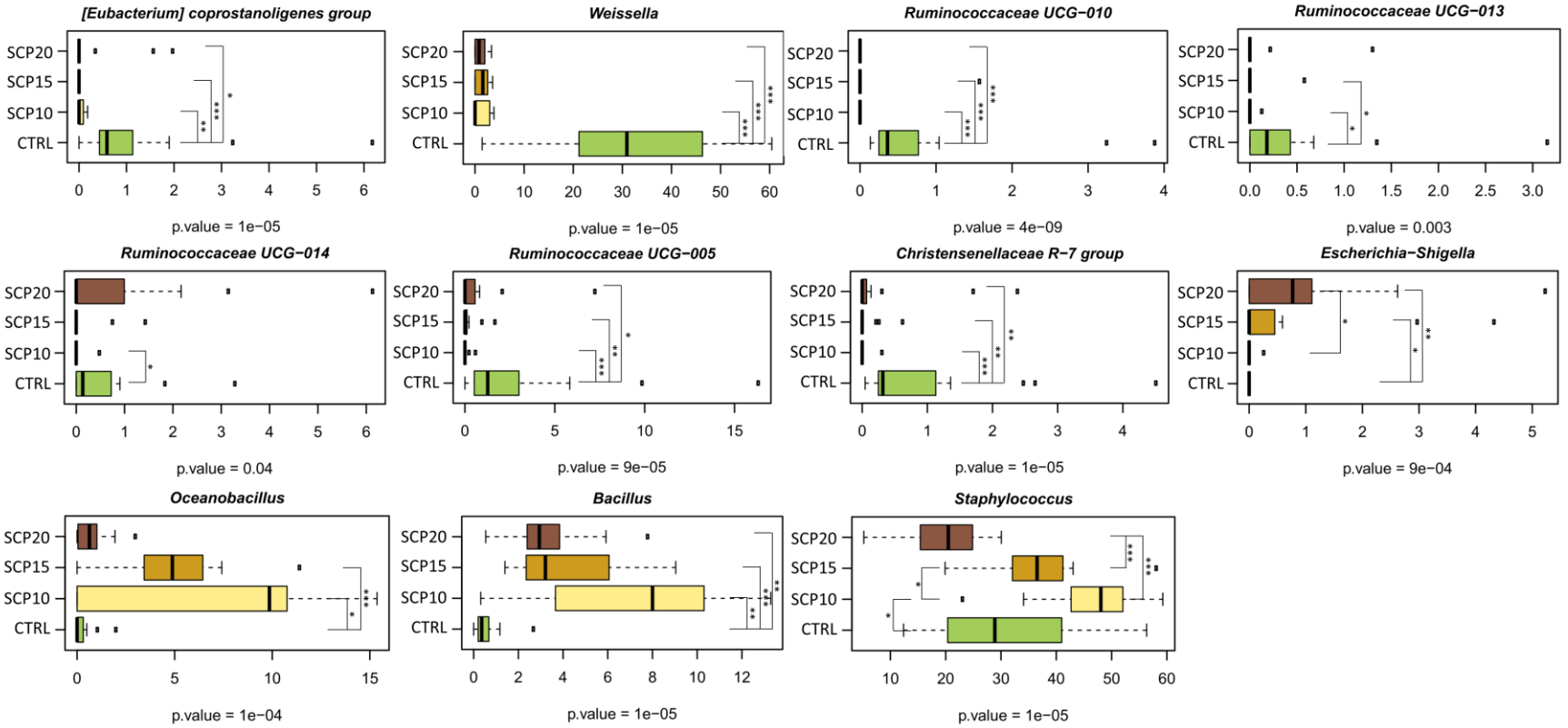

A
Relative Abundance - Phylum
100
80
60
40
20
0
CTRL
SCP10
SCP15
SCP20
Other unclassified
Other Phyla
Verrucomicrobia
Tenericutes
Proteobacteria
Planctomycetes
Firmicutes
Cyanobacteria
Bacteroidetes
Actinobacteria
Acidobacteria
B
Relative Abundance - Family
Other unclassified
Other Families
Verrucomicrobiaceae
Mycoplasmataceae
Vibrionaceae
Pseudomonadaceae
Moraxellaceae
Hahellaceae
Enterobacteriaceae
Comamonadaceae
Sphingomonadaceae
Rhodospirillaceae
Rhodobacteraceae
Rhizobiaceae
Methylobacteriaceae
Bradyrhizobiaceae
Planctomycetaceae
Veillonellaceae
Ruminococcaceae
Peptostreptococcaceae
Peptococcaceae
Lachnospiraceae
Family XI
Clostridiales vadinBB60
Clostridiaceae 1
Christensenellaceae
Streptococcaceae
Leuconostocaceae
Lactobacillaceae
Enterococcaceae
Carnobacteriaceae
Staphylococcaceae
Planococcaceae
Paenibacillaceae
Bacillaceae
FamilyI
Flavobacteriaceae
Flammeovirgaceae
Rikenellaceae
Prevotellaceae
Porphyromonadacea
Bacteroidaceae
Coriobacteriaceae
Micrococcaceae
Dermabacteraceae
Brevibacteriaceae
Corynebacteriaceae
Bifidobacteriaceae

Figure 4


[Eubacterium] coprostanoligenes group
SCP20
SCP15
SCP10
CTRL
0 1 2 3 4 5 6
p.value = 1e−05
Weissella
0 10 20 30 40 50 60
p.value = 1e−05
Ruminococcaceae UCG−010
0 1 2 3 4
p.value = 4e−09
Ruminococcaceae UCG−013
0.0 0.5 1.0 1.5 2.0 2.5 3.0
p.value = 0.003
Ruminococcaceae UCG−014
0 1 2 3 4 5 6
p.value = 0.04
Ruminococcaceae UCG−005
0 5 10 15
p.value = 9e−05
Christensenellaceae R−7 group
0 1 2 3 4
p.value = 1e−05
Escherichia−Shigella
0 1 2 3 4 5
p.value = 9e−04
Oceanobacillus
0 5 10 15
p.value = 1e−04
Bacillus
0 2 4 6 8 10 12
p.value = 1e−05
Staphylococcus
10 20 30 40 50 60
p.value = 1e−05