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Microalgae and phytase dietary supplementation improved growth and gut microbiota in juvenile European seabass (*Dicentrarchus labrax*)

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Abstract

Fishmeal and fish oil have been the main sources of protein and fatty acid for aquaculture fish. However, their increasing price and low sustainability have led the aquafeed industry to seek sustainable alternative feedstuffs to meet the nutritional requirements of fish and improve their health and performance. Plant proteins have been successfully used to replace fishery derivatives in aquafeeds, but the presence of anti-nutritional substances is a potential drawback of this approach. Thus, it has been reported that phytate breakdown can be caused by feed supplementation with exogenous phytase. The inclusion of microalgae has been proposed to improve gut functionality in fish fed diets with a high vegetable protein content. The aim of this study was to evaluate the effect on the growth and gut microbiota of European seabass (*Dicentrarchus labrax*) juveniles of a diet containing a blend of microalgae (*Arthrospira platensis* and *Nannochloropsis gaditana*) and different concentrations of phytase. An 83-day feeding trial was conducted, comprising four experimental diets with 2.5% microalgae and 500, 1,000, 2,000, or 10,000 phytase units (FTU)/kg feed and a microalgae- and phytase-free control diet. At the end of the trial, a significantly increased body weight was observed in fish fed the diet with the highest phytase concentration (10,000 FTU/kg) *versus* controls, although the gut bacterial composition did not differ from controls in alpha or beta diversity with either majority (Weighted UniFrac) or minority bacterial strains (Unweighted UniFrac). In comparison to the control group, the groups fed diets with 1,000 or 2,000 FTU/kg diets had a lower alpha diversity (Shannon's diversity index), while those fed diets with 500 FTU/kg or 1,000 FTU/kg showed distinct clusters in beta diversity (involving minority ASVs). According to these findings, the diet containing the 2.5% microalgae blend with 10,000 FTU/kg may be useful to increase the aquafeed quality and sustain the growth performance of juvenile European seabass.

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Introduction

The nutritional requirements of aquaculture fish have traditionally been met by using fishmeal and fish oil as the main sources of proteins and fatty acids [1]; however, their increasing price and low sustainability have led the aquafeed production industry to seek alternatives [2]. Various ingredients have been proposed to fully or partially replace fishmeal and fish oil in aquafeeds [3–6]. Microalgae have recently attracted attention as a functional ingredient at a low dietary inclusion level [7, 8]. In particular, certain strains of the phylum *Cyanobacteria* have been described as a sustainable alternative to cover the nutritional requirements of fish and improve their health and performance [7, 9–12]. However, the nutritional value of microalgae can vary substantially among different algal species and according to the method employed to incorporate the algal biomass into functional aquafeed (FAO, 2019).

Numerous studies have demonstrated the benefits of microalgae biomass as feed ingredient for aquaculture [12], based on their nutritional quality and positive effects on fish growth [11, 13, 14]. The inclusion of microalgae has been found to increase the muscle content of proteins, triglycerides, and omega-3 fatty acids and to improve the physiological activity and carcass quality of fish [15]. The genera of microalgae most frequently used in aquaculture feed include *Tetraselmis*, *Schizochytrium*, *Chlorella*, *Nannochloropsis*, and *Arthrospira* [reviewed in 12, 16]. *Arthrospira* are known to offer a high content of proteins, vitamins, minerals, and active compounds and balanced amino acid and fatty acid profiles [17]. Specifically, *A. platensis* has been successfully used in aquafeed for gilthead seabream (*Sparus aurata*), rainbow trout (*Onchorhynchus mykiss*), and pabda catfish (*Ompok pabda*) [7, 18, 19]. *Nannochloropsis* species also have a high protein, vitamin, pigment, and fatty acid content and are widely used in the marine aquaculture nutrition of gilthead seabream, especially during larval stages [20]. The dietary inclusion of *N. gaditana* was found to increase the body length and body weight of gilthead seabream (*S. aurata*) [11] and enhance the bacterial species richness [20]. Nevertheless, the inclusion of microalgae in aquafeeds may be more problematic for larvae, fry, and juveniles due to their lower enzymatic activity in comparison to adult fish and their lesser capability to digest microalgae-formulated diets [21].

Phytase hydrolyzes indigestible phytate, making the phosphorus available for intestinal absorption [22], and supplementation with this enzyme increases the bioavailability of other minerals and trace elements [22]. Hence, the incorporation of commercial enzymes such as phytase in aquafeeds may improve the digestibility and bioavailability of nutrients [23]. In their study of rainbow trout (*O. mykiss*), Cheng et al. [24] reported that dietary supplementation with microbial phytase increased the digestibility of barley, canola meal, and wheat-based diets. In other studies, the inclusion of microbial phytase in plant-based diets improved apparent nutrient digestibility but did not influence the growth performance or body composition of olive flounder (*Paralichthys olivaceus*) or Korean rockfish (*Sebastes schlegeli*) juveniles [25, 26]. In a study of red seabream (*Pagrus major*), dietary supplementation with soybean and 2,000 phytase units (FTU) kg feed showed an increase in productive parameters [27]. However, to our best knowledge, no study has been published on the potential effects of dietary supplementation with exogenous phytase plus microalgae on the intestinal microbiota and growth performance of aquaculture fish.

The aim of this study was to determine the effect of dietary supplementation with a blend of microalgae (*A. platensis* and *N. gaditana*) plus 500, 1,000, 2,000, or 10,000 FTU/kg feed on the intestinal microbiota and growth performance of European seabass (*D. labrax*) juveniles. The study hypothesis was that the combination of phytase and microalgae would enhance nutrient digestibility and bioavailability, and that the changes in gut microbiota would increase their final body weight.

Materials and methods

Animals, experimental design and fish sampling

European seabass (*Dicentrarchus labrax*) juveniles (n=375) from a commercial company (Cultivos Piscícolas de Barbate S.L., Cádiz, Spain) were randomly distributed among 15 tanks of 400 L (25 fish per tank with initial stocking density of ~2.5 kg of fish/m^3) in an open system circuit. Fish were acclimated for one month to seawater (38‰ salinity) and the natural photoperiod (September-December) at our latitude (36°35'06"N; 06°13'48"W; Cádiz, Spain) under a constant temperature of 18–19 °C. Tanks were located in indoor wet laboratories at the *Servicios Centrales de Investigación en Cultivos Marinos* (see Ethical Approval and Standards section).

Microalgae cultures were up-scaled from Erlenmeyer flasks with F/2 nutrient medium for seawater strains and BG11 medium for freshwater strains at a mean light intensity of 240 µmol photons m^{-2} s^{-1}, photoperiod of 12:12 (L: D), and temperature of 25±2 °C, providing a continuous supply of 1.5% CO_2 enriched air during the light period. Crude biomass of *A. platensis* was produced

in 100 L bubble columns in a greenhouse at the Biorizon Biotech facilities (Almería, Spain), while crude biomass of *N. gaditana* was obtained in tubular photobioreactors at the pilot plant (EU H2020 SABANA facilities) of the University of Almeria (Spain). The culture was harvested daily by centrifugation (dilution rate of 0.3 d^{-1}). Raw microalgae biomass (approx. 15% dry matter) was freeze-dried and stored at -20 ºC until utilization. On a dry matter basis, the crude biomass was 65% crude protein and 5% crude lipid for *A. platensis* and 44.5% crude protein and 17.7% crude lipid for *N. gaditana*.

Table 1 Ingredients (g/kg on dry matter) of the diets administered to juvenile European seabass, including a control diet and experimental diets supplemented with 2.5% microalgae (*A. platensis* and *N. gaditana*) and different concentrations of phytase (500, 1,000, 2,000, or 10,000 FTU/kg)

Ingredient composition	Control	2.5% microalgae + phytase
LT94 fishmeal [1]	100.0	100.0
Squid meal [2]	20.0	20.0
CPSP90 [3]	10.0	10.0
Krill meal [4]	20.0	20.0
Microalgae blend [5]		25.0
Wheat gluten [6]	80.0	80.0
Soybean protein concentrate [7]	330.0	330.0
Pea protein concentrate [8]	80.0	80.0
Sunflower seed meal [9]	125.0	125.0
Fish oil [10]	60.0	60.0
Soybean oil	60.0	60.0
Soybean lecithin [11]	10.0	10.0
Wheat meal [12]	34.5	9.5
Choline chloride [13]	5.0	5.0
Betaine [14]	5.0	5.0
Vitamin & mineral premix [15]	20.0	20.0
Vitamin C [16]	1.0	1.0
Lysine [17]	12.0	12.0
Methionine [18]	6.0	6.0
Tryptophan [18]	1.5	1.5
Guar gum [19]	20.0	20.0

[1] 69.4% crude protein, 12.3% crude lipid (Norsildemel, Bergen, Norway). [2, 3,4] purchased from Bacarel (UK). CPSP90=enzymatically pre-digested fishmeal. [5] blend of marine (*N. gaditana*) and freshwater (*A. platensis*) microalgae (1:1): Total P: 1.12 g and 0.9 g 100 g; total phytate P: 0.01 g and 0.01 g 100 g in *N. gaditana* and *A. platensis*, respectively. [6] 78% crude protein (Lorca Nutrición Animal SA, Murcia, Spain). Total P: 0.718 g 100 g; total phytate P: 0.01 g 100 g. [7] Soycomil, 60% crude protein, 1.5% crude lipid (ADM, Poland). Total P: 0.66 g 100 g; total phytate P: 0.42 g 100 g. [8] Pea protein concentrate, 85% crude protein, 1.5% crude lipid (Emilio Peña SA, Spain). [9] Sunflower seed meal, 35% crude protein, 6% crude lipid (LorcaNutrition, Spain). Total P: 0.77 g 100 g; total phytate P: 0.33 g 100 g. [10] AF117DHA (Afamsa, Spain). [11] P700IP (Lecico, DE). [12] Local supplier (Almería, Spain). [13, 14, 17, 18] Lorca Nutrición Animal SA (Murcia, Spain). [15]*Lifebioencapsulation* SL (Almería, Spain). Vitamins (mg kg^{-1}): vitamin A (retinyl acetate), 2,000,000 UI; vitamin D3 (DL-cholecalciferol), 200,000 UI; vitamin E (Lutavit E50), 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2,500 mg; vitamin B1(thiamine hydrochloride), 3,000 mg; vitamin B2 (riboflavin), 3,000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2,000 mg; vitamin B9 (folic acid), 1,500 mg; vitamin B12 (cyanocobalamin), 10 mg; vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine (Betafin S1), 50,000 mg. Minerals (mg kg-1): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 18.6%; (186,000 mg); KCl, 2.41%; (24,100 mg); NaCl, 4.0% (40,000 mg), excipients: colloidal silica. [16,17,18] TECNOVIT, Spain. [19] EPSA, Spain. [20] ePhyt® from Global Feed S.L. (Tervalis Group, Huelva, Spain). 1309.9 phytase activity units (FTU)/mL. One FTU is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per min under the reaction conditions specified in International Standard ISO30024

Five isolipid and isoenergetic experimental diets (49% crude protein, 15% crude lipid) were designed and formulated by the Experimental Diet Service of the University of Almeria (Spain). The proximate composition of the diets is exhibited in Table 1. The experimental diets contained 2.5% crude biomass of *A. platensis* and *N. gaditana* plus 500, 1,000, 2,000, or 10,000 FTU/kg feed, defining I FTU as the amount of enzyme that releases 1 µmol of inorganic phosphate per min from phytate under the reaction conditions specified in International Standard ISO 30024:2009. A microalgae- and phytase-free diet served as control diet. The phytase used was ePhyt® (Global Feed S.L. Tervalis Group, Huelva, Spain), a bacterial 3-phytase preparation (EC 3.1.3.8.) produced by fermentation of a strain of *Komagataella phaffii*. It is classified as a 'zootechnical additive' belonging to the functional group of 'digestibility enhancers', and it is authorized by the European Union (EC 1831/2003) as an additive for animal nutrition (additive no. 4a25).

Before starting the feeding trial, fish were anesthetized with 30 ppm of clove oil and weighed (mean initial body weight [BW] of 29.1±0.2 g). They were then randomly housed in the tanks to obtain a similar initial biomass in each tank, adjusting the volume to maintain a density of 2.5 kg of fish/m^3 throughout the experiment. Each dietary treatment was tested in triplicate, the minimum number of tanks required for aquaculture research. Fish were fed *ad libitum* twice daily (at 10:00 and 17:00) during the 83-day experimental period. The water in all tanks was oxygen-saturated (>90% O_2 saturation) using air stones. Water concentrations of ammonia (<0.1 mg/L), nitrite (<0.2 mg/L), and nitrate (<50 mg/L) were determined daily at 09:00 using commercial kits (SERA® GmbH, Heinsberg, Germany). On day 83, fish were fasted overnight for weight measurement, and four fish per tank (12 fish per experimental group, 60 fish for the whole assay) were then euthanized with a lethal dose (1 mL/L of marine water) of 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO). These 60 fish were immediately dissected, using sterile material to collect whole intestines and store them in sterile containers, which were kept at -80 ºC until DNA extraction.

DNA extraction

DNA extraction of the whole intestine of European seabass juveniles followed the Modified Salting Out Procedure (MSOP) by Martín-Platero et al. [28], which includes an initial mechanical lysis step with a FastPrep

FP120 cell disrupter (BIO 101, Thermo Savant, Waltham, MA) to increase cell lysis. Briefly, intestines were placed in a 2 mL microcentrifuge screw cap tube filled with 100 mg of 2 mm zirconia beads and were homogenized by two consecutive pulses of 30 s at speed 5 in FastPrep FP120, followed by completion of the MSOP protocol. The DNA extraction yield was assessed by 0.7% agarose gel electrophoresis, and the DNA concentration was measured with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). DNA was stored at -20 ºC until PCR amplification.

V6-V8 16 S rRNA gene amplification and high-throughput sequencing

Gene libraries based on the V6-V8 region of 16 S rRNA were constructed using the primer pairs B969F (5'-ACGCGHNRAACCTTACC-3') and BA1406R (5'-ACGGGCRGTGWGTRCAA-3') (Comeau et al., 2011) with Illumina adapter overhang sequences. Amplicons were generated with the iProof™ High-Fidelity DNA Polymerase kit (BioRad®, Hercules, CA). The purified PCR products were used as template for a second PCR in which two unique Illumina compatible barcodes were indexed to each sample. These barcodes allow the derived sequences to be demultiplexed into their respective samples in downstream analysis. The barcodes overlapped with the sequence of the primers used in the first PCR. All purification steps used DNA Purification SPRI Magnetic Beads (Canvax®, Valladolid, Spain) according to the manufacturer's instructions. PCR amplicons were assessed by 1% agarose gel electrophoresis, and DNA concentrations were measured using a Qubit® 3.0 Fluorometer (Invitrogen™, Carlsbad, CA). Next, PCR amplicons were pooled in equimolar concentrations. High-throughput sequencing was carried out with the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), resulting in paired-end reads of 2×300 bp length. Sequencing was done on the Illumina MiSeq platform at the "López-Neyra" Institute of Parasitology and Biomedicine (Granada, Spain).

Sequences processing and data analysis

The 16 S rRNA sequences generated by Illumina MiSeq were analyzed with Quantitative Insights Into Microbial Ecology (QIIME2 v2021.4) [29] software. First, primer trimming was performed using *cutadapt* plugin default parameters [30], and pair reads were joined using default parameters. A quality filter was then applied to exclude sequences that had more than three consecutive base pairs with a Phred score below 20. Sequencing errors were avoided by using Deblur Amplicon Sequence Variants (ASVs), a sub-operational-taxonomic-unit approach [31]. Fragment insertion of 16 S sequences was performed using the SEPP algorithm implemented in QIIME2 to align sequences and construct the bacterial phylogenetic tree [32]. Taxonomy assignation was based on Greengenes 13.8 with a similarity of 99% [33]. Finally, chloroplast, mitochondria, and non-bacterial reads were filtered out.

Statistics

Fish were weighed at the beginning and end of the experiment but were not individually tagged; therefore, the tank was used as sampling unit in GLMs to evaluate the effect of treatments (fixed factor) on fish weight and growth performance. The following parameters were then determined for each tank: (i) specific growth rate = (100 × (natural logarithm final body weight - natural logarithm initial body weight)/days; (ii) weight gain = (100 × (body weight increase)/initial body weight; and (iii) feed efficiency=weight gain/total feed intake.

For alpha diversity analysis, the ASV table was rarefied at a depth of 10,000 sequences per sample, excluding samples that did not reach this depth from further analyses (i.e., 1 from control, 1 from 500 FTU/kg, 2 from 2,000 FTU/kg, and 2 from 10,000 FTU/kg groups). TheASV table was used to calculate Shannon's diversity index [34], Faith's Phylogenetic Diversity index [35], and the ASV richness (number of ASVs). Generalized Linear Mixed Models (GLMMs) were constructed to explore the effect of diet as fixed factor and tank nested in diet as random factor on these alpha diversity indices, considering individual fish as the experimental unit. Fisher's LSD test was used for *post-hoc* comparisons.

All weight and growth parameters followed a Gaussian distribution (Kolmogorov-Smirnov, $p>0.268$). When the treatment proved statistically significant in the GLMM, Fisher's LSD test was performed to evaluate differences between treatment levels. STATISTICA 12.0 (StatSoft) was used to analyze data on weight, growth, and alpha diversity indices.

Weighted and Unweighted UniFrac beta diversity distance matrices [36, 37] were calculated from the rarefied ASV table (depth of 10,000 sequences per sample). Permutational ANOVAs were performed to assess the effect of diet as fixed factor and tank nested in diet as random factor on the two UniFrac distance matrices, using PRIMER-7 software (PRIMER-e) implemented with the PERMANOVA plugin. Principal Coordinate Analyses (PCoAs) were conducted to visualize the two first axes using EMPeror 2021.4.0 [38].

Results

Effect of diet on growth performance of the European seabass juveniles

The initial mean body weight of the fish did not differ among treatment groups at the beginning of the feeding trial (Table 2). However, the final mean body weight

Table 2 One-way ANOVA on the effects of diet on European seabass juveniles fed with control diet or diet supplemented with microalgae (*A. platensis* and *N. gaditana*) and different phytase units (FTU/kg). Tanks served as experimental units. D.f. = degree of freedom, the first number being the d.f. of the independent variable and the second the d.f. of the error term. Significant *p*-values are shown in bold. Letters with different superscript letters in the same row denote significant differences (Fisher LSD Posthoc test; $p < 0.05$)

	Control	2.5% microalgae + 500 FTU/kg	2.5% microalgae + 1,000 FTU/kg	2.5% microalgae + 2,000 FTU/kg	2.5% microalgae + 10,000 FTU/kg	D.f.	F	*P*
Initial mean body weight (g/fish)	28.84 ± 0.03^{a}	29.26 ± 0.26^{a}	29.38 ± 0.15^{a}	29.16 ± 0.29^{a}	28.86 ± 0.43^{a}	4,10	0.82	0.543
Final mean body weight (g/fish)	66.76 ± 1.26^{a}	70.18 ± 1.30^{a}	69.80 ± 1.58^{a}	72.19 ± 1.04^{a}	75.51 ± 1.01^{b}	4,10	6.65	**0.007**
Weight gain (%)	131.5 ± 4.2^{a}	139.8 ± 2.4^{a}	137.6 ± 5.9^{a}	147.6 ± 2.2^{ab}	161.8 ± 5.4^{b}	4,10	7.34	**0.005**
Specific growth rate	1.00 ± 0.02^{a}	1.04 ± 0.01^{a}	1.03 ± 0.03^{a}	1.08 ± 0.01^{ab}	1.15 ± 0.02^{b}	4,10	7.15	**0.006**
Feed efficiency	0.67 ± 0.01^{a}	0.70 ± 0.02^{a}	0.71 ± 0.03^{ab}	0.72 ± 0.02^{ab}	0.80 ± 0.02^{b}	4,10	5.61	**0.012**
Survival (%)	97.3 ± 1.3^{a}	97.3 ± 1.3^{a}	97.3 ± 2.7^{a}	96.0 ± 2.3^{a}	98.7 ± 1.3^{a}	4,10	0.25	0.903

was significantly higher in the group receiving 2.5% microalgae plus 10,000 FTU/kg than in any other group. The final body weight of the groups fed diets with 2.5% microalgae plus 500, 1,000, and 2,000 FTU/kg did not differ with those in the control group, showing intermediate body weight values (Table 2).

As shown in Table 2, the weight gain and specific growth over the 83-day experimental period were also significantly higher in the group fed 2.5% microalgae plus 10,000 FTU/kg than in the control group or the groups fed 2.5% microalgae plus 500 or 1,000 FTU/kg (Fisher LSD test, $p=0.036$). Likewise, the feed efficiency was significantly higher in fish fed 2.5% microalgae plus 10,000 FTU/kg than in the control group or the group fed 2.5% microalgae plus 500 FTU/kg (Fisher LSD test, $p=0.038$). The survival rate did not significantly differ between treatment groups (Table 2).

Bacterial community composition

The gut microbiota of the juvenile European seabass was dominated by *Gammaproteobacteria* (>60%), followed by *Alphaproteobacteria*, *Bacilli*, *Actinobacteria*, and *Betaproteobacteria*. The relative abundance of these classes varied between controls and the groups fed 2.5% microalgae plus 500, 1,000, or 2,000 FTU/kg, while the microbial composition of the gut microbiota was similar between controls and the group fed 2.5% microalgae plus 10,000 FTU/kg (Fig. 1).

The genera dominating the gut microbiota in the control group were *Pseudomonas*, *Vibrio*, and *Ochrobactrum*. An increase in *Vibrio* and *Ochrobactrum* and a decrease in minority genera were observed in fish fed 2.5% microalgae plus 500, 1,000, or 2,000 FTU/kg (Fig. 2), whereas the bacterial composition was again similar between controls and the group fed 2.5% microalgae plus 10,000 FTU/kg.

Effect of feeding diet on alpha and beta diversity

There were no statistically significant differences in ASV richness or Faith's phylogenetic diversity index between treatment groups at the end of the feeding trial, although marginally significant differences were observed between experimental groups (Table 3). However, Shannon's diversity index values (Table 3) were significantly lower in the groups fed 2.5% algae plus 1,000 or 2,000 FTU/kg than in the control group or the group fed 2.5% algae plus 10,000 FTU/kg (Fisher LSD Post-hoc, $p<0.028$), with no difference between the last two groups (Fisher LSD Post-hoc, $p=0.604$, Fig. 3). As depicted in Fig. 3, intermediate values were observed for the group fed 5% algae plus 500 FTU/kg.

At the end of the 83-day feeding trial, the composition of gut microbiota differed between treatment groups (Fig. 4; Table 4). Significant differences in majority ASVs (Weighted Unifrac) were found between the group fed 500 FTU/kg and the group fed 1,000 FTU/kg and between the group fed 500 FTU/kg and the group fed 10,000 FTU/kg (pairwise test, $t>2.25$, $P<0.047$). The composition of the bacterial community did not significantly differ between the control group and any experimental group (pairwise test, $t<2.00$, $P>0.080$). However, pairwise comparisons revealed significant differences in minority ASVs (Unweighted UniFrac) between the group fed 500 FTU/kg and the group fed 1,000 FTU/kg (pairwise test, $t>1.12$, $p=0.048$) and between these two groups and the other experimental groups but not between the control, 2,000 FTU/kg, and 10,000 FTU/kg groups (pairwise test, $t<0.99$, $p>0.473$).

Discussion

This study contributes to the search for sustainable alternatives to increasingly costly fishmeal and fish oil-based aquafeeds [39, 40] for aquaculture in the context of rising aquaculture production [41]. Dietary supplementation with different species of microalgae has been found to exert positive effects on fish health and performance at different fish rearing stages [42]. After this 38-day feeding trial of juvenile European seabass fed a blend of microalgae (*A. platensis* and *N. gaditana*) with different phytase concentrations, the body weight was significantly

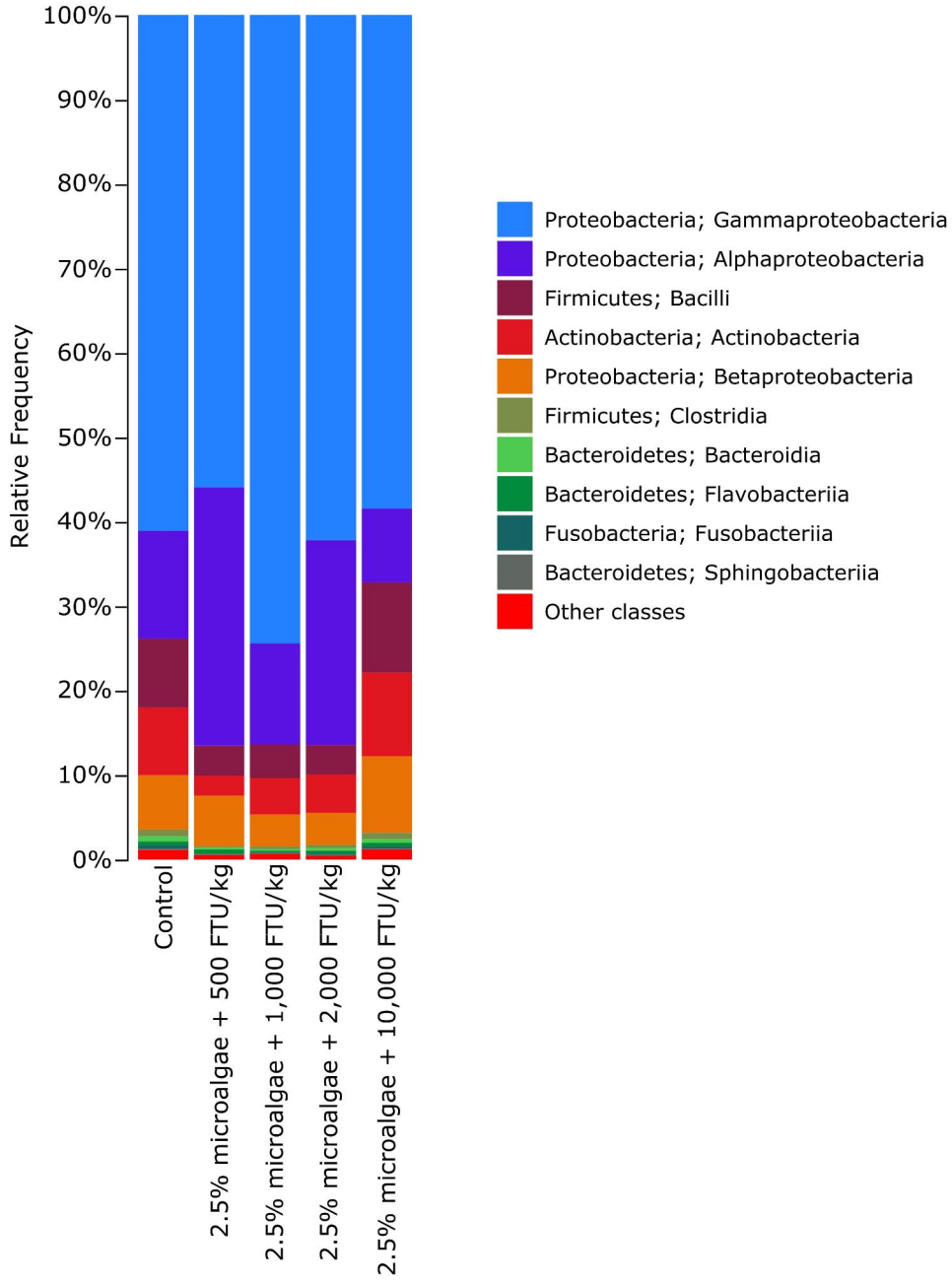

Fig. 1 Microbial composition at class level of juvenile European seabass (*Dicentrarchus labrax*) gut microbiota grouped by experimental diet (2.5% microalgae [*A. platensis* and *N. gaditana*] plus 500, 1,000, 2,000, or 10,000 FTU/kg phytase). Each bar represents the mean relative abundance of the different bacterial classes in samples from each group. Classes in the legend are ordered from the most to least abundant

higher in fish fed the highest concentration (10,000 FTU/kg) than in controls on an algae- and phytate-free diet. Furthermore, there was no difference between these two groups in gut bacterial composition, considering alpha or beta diversity with either majority (Weighted UniFrac) or minority bacterial strains (Unweighted UniFrac). In comparison to controls, fish fed diets with 1,000 or 2,000 FTU/kg had a lower alpha diversity (Shannon's diversity index) and those fed diets with 500 FTU/kg or 1,000 FTU/kg clustered significantly apart in beta diversity analyses (involving minority ASVs).

Supplementation with a blend of microalgae did not have negative effects on the growth of these fish, in agreement with previous reports. In one study, the inclusion of *N. gaditana* extracts did not change final body weight but improved fish muscle composition and skin pigmentation in gilthead seabream (*S. aurata*) juveniles [43]. Dietary supplementation with microalgae has been

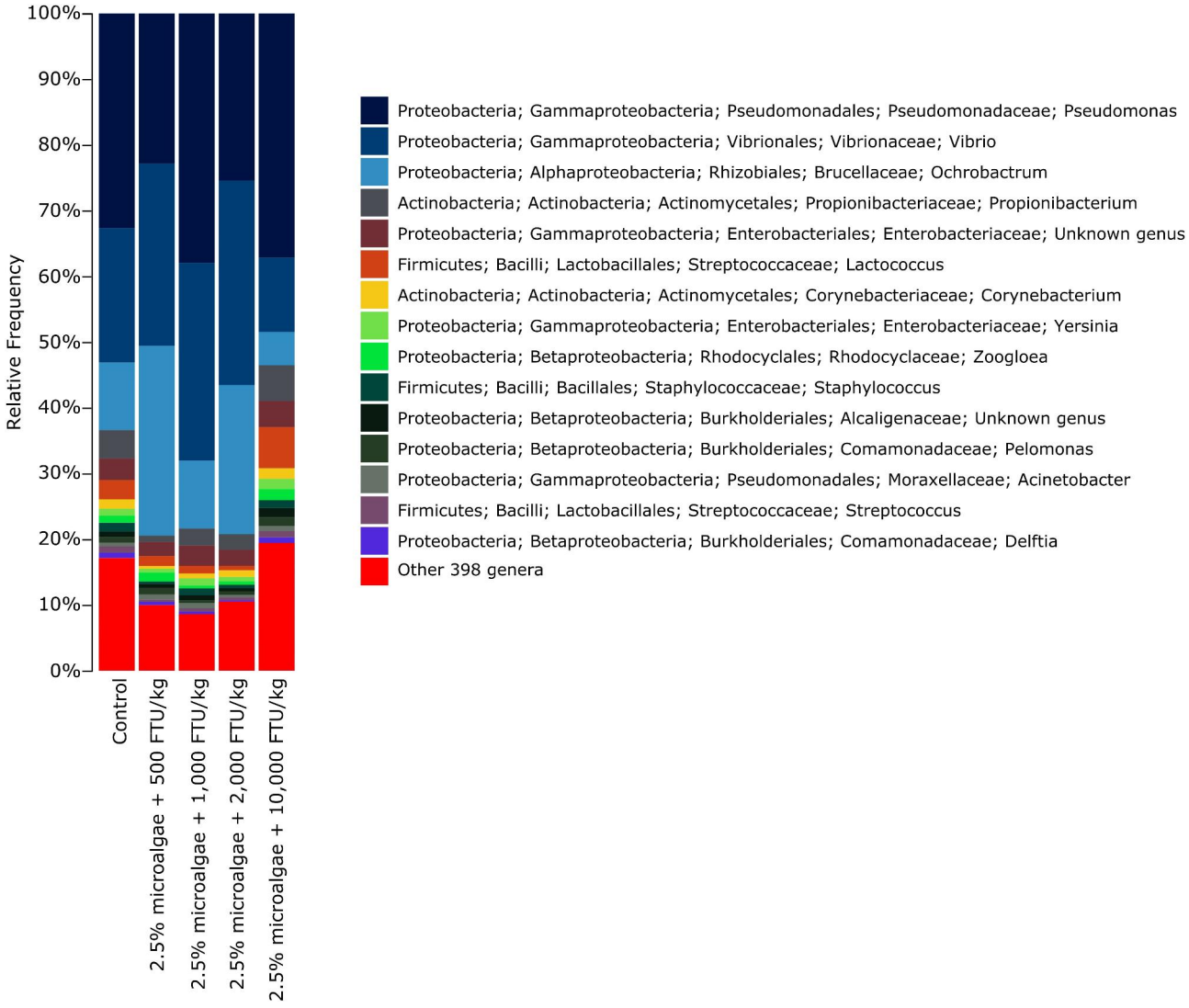

Fig. 2 Microbial composition at genus level of juvenile European seabass (*Dicentrarchus labrax*) gut microbiota group by experimental diet (2.5% microalgae [*A. platensis* and *N. gaditana*] plus 500, 1,000, 2,000, or 10,000 FTU/kg of phytase). Each bar represents the mean relative abundance of the different bacterial genera in samples from each group. Genera in the legend are ordered from the most to least abundant

Table 3 General Linear mixed models on the effects of fish diet (control, 500, 1,000, 2,000 and 10,000 FTU/kg) and tank nested in diet on alpha diversity indices of the gut microbiota of juvenile European seabass. D.f. = degree of freedom, the first number being the d.f. of the independent variable and the second the d.f. of the error term. Significant p-values ($p < 0.05$) are indicated in bold

Alpha Diversity Index	Explanatory variables	D.f	F	*P*
Shannon	Diet	4,39	14.85	**<0.001**
	Tank (Diet)	10,39	0.23	0.598
Faith PD	Diet	4,39	2.95	0.070
	Tank (Diet)	10,39	0.63	0.152
Bacterial richness	Diet	4,39	2.66	0.089
	Tank (Diet)	10,39	0.60	0.800

found to increase the body weight and body length of seabream (*S. aurata*) [44] and to improve the growth performance, feed efficiency, and/or health status of gilthead seabream (*S. aurata*) juveniles close to commercial size [45] and of Nile tilapia (*Oreochromis niloticus*) [46] and Senegalese sole (*Solea senegalensis*) [44] juveniles. In the same line, low dietary supplementation with *A. platensis* biomass (from 2% up to 10%) was found to cause positive changes in digestive enzyme activities and gut morphology in seabream (*S. aurata*) [7, 10]. In a previous study of European seabass, similar growth and feed conversion rates were observed between fish fed diets with microalgae and those fed conventional diets, with both groups maintaining good morphological intestinal histomorphology [47–49].

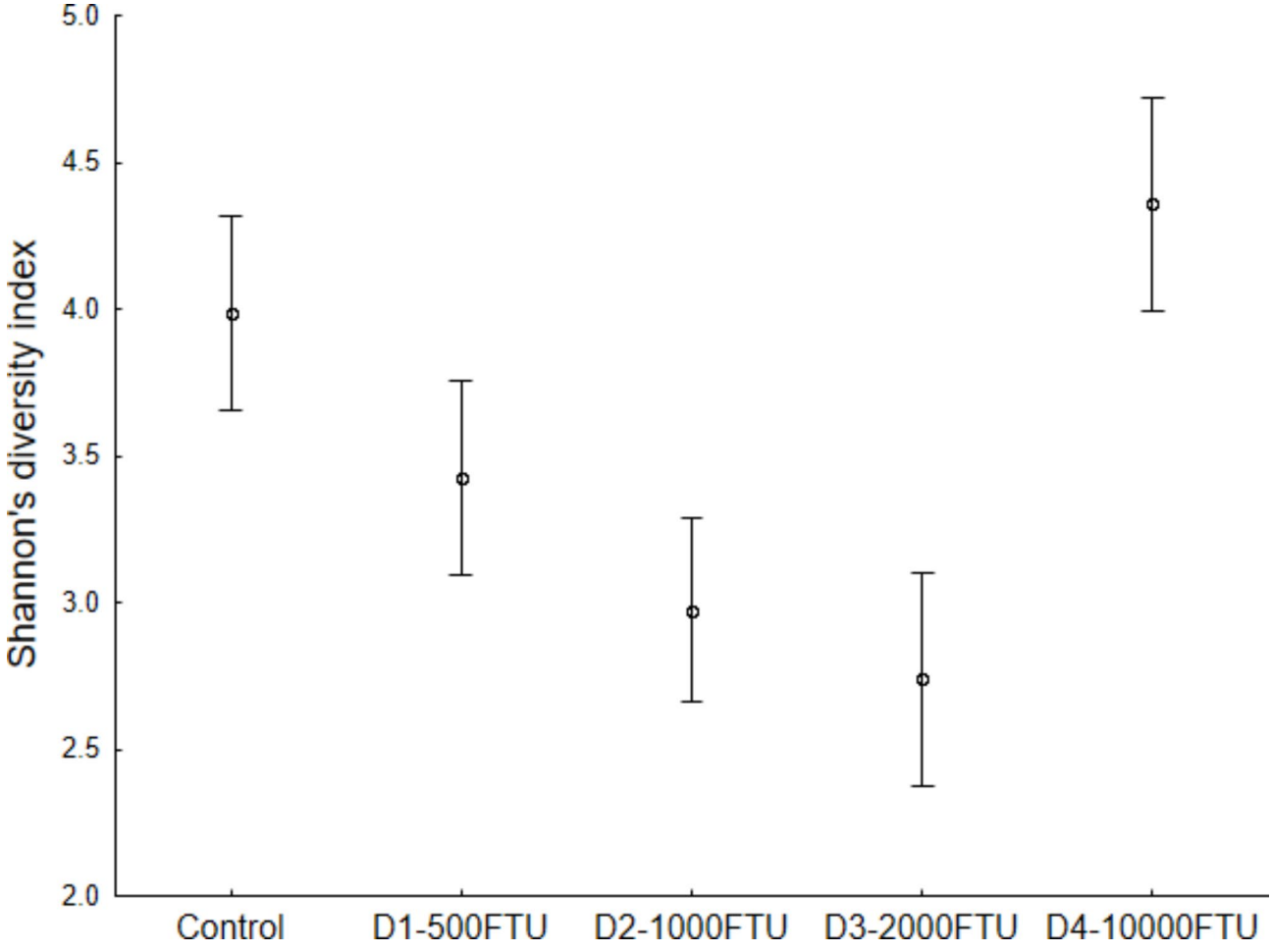

Fig. 3 Shannon's diversity index of the gut microbiota of juvenile European seabass (*Dicentrarchus labrax*) fed with control diet or a diet supplemented with 2.5% microalgae (*A. platensis* and *N. gaditana*) and different concentrations of phytase (500, 1,000, 2,000, or 10,000 FTU/kg). Bars indicate the standard error of the mean

However, protein assimilation problems have been reported in carnivorous fish species receiving feed based on plant proteins, reviewed in [50]. These include an unbalanced amino acid profile, a deficiency in highly unsaturated fatty acids, and the presence of anti-nutritional compounds such as phytate and non-starch polysaccharides [5, 51]. These compounds are found in diets rich in plant ingredients and can produce changes in the enzymatic activity and gut physiology of aquaculture fish [5, 51]. Hence, the design of plant-based diets for carnivorous fish such as the European seabass should take these potential drawbacks into account. For instance, dietary supplementation with phytase can improve the nutrient bioavailability of plant-based feeds, especially algal biomasses, increasing feed digestibility by enhancing the availability of phosphorus from phytate [22, 52]. This improved bioavailability of phosphorus has been found to reduce its discharge into the aquatic environment as well as increasing the weight gain and phosphorus utilization of fish, augmenting their carcass protein content, improving their protein retention, and enhancing their immune response [53, 54], as also demonstrated in European seabass [55].

Various authors have described the benefits of phytase for feed utilization and nutrient digestibility in aquaculture diets [reviewed in 22, and 23]. In this way, phytase dietary supplementation increased productive performance in red seabreams (*P. major*) [27], rainbow trout (*O. mykiss*) [56], and Nile tilapias (*O. niloticus*) [54]. Previous studies confirmed the beneficial effects of dietary phytase in European seabass juveniles, which only showed a preference towards a plant-based diet when it contained phytase [57]. Moreover, European seabass fed a plant-based diet that included phytase showed improvements in growth performance, hematological status, immune response, and gut and liver histology [58–60]. These positive effects were also observed in the present study, especially in the group receiving 2.5% microalgae (*A. platensis* and *N. gaditana*) plus 10,000 FTU/kg, which evidenced a significant increase in body weight, specific growth rate,

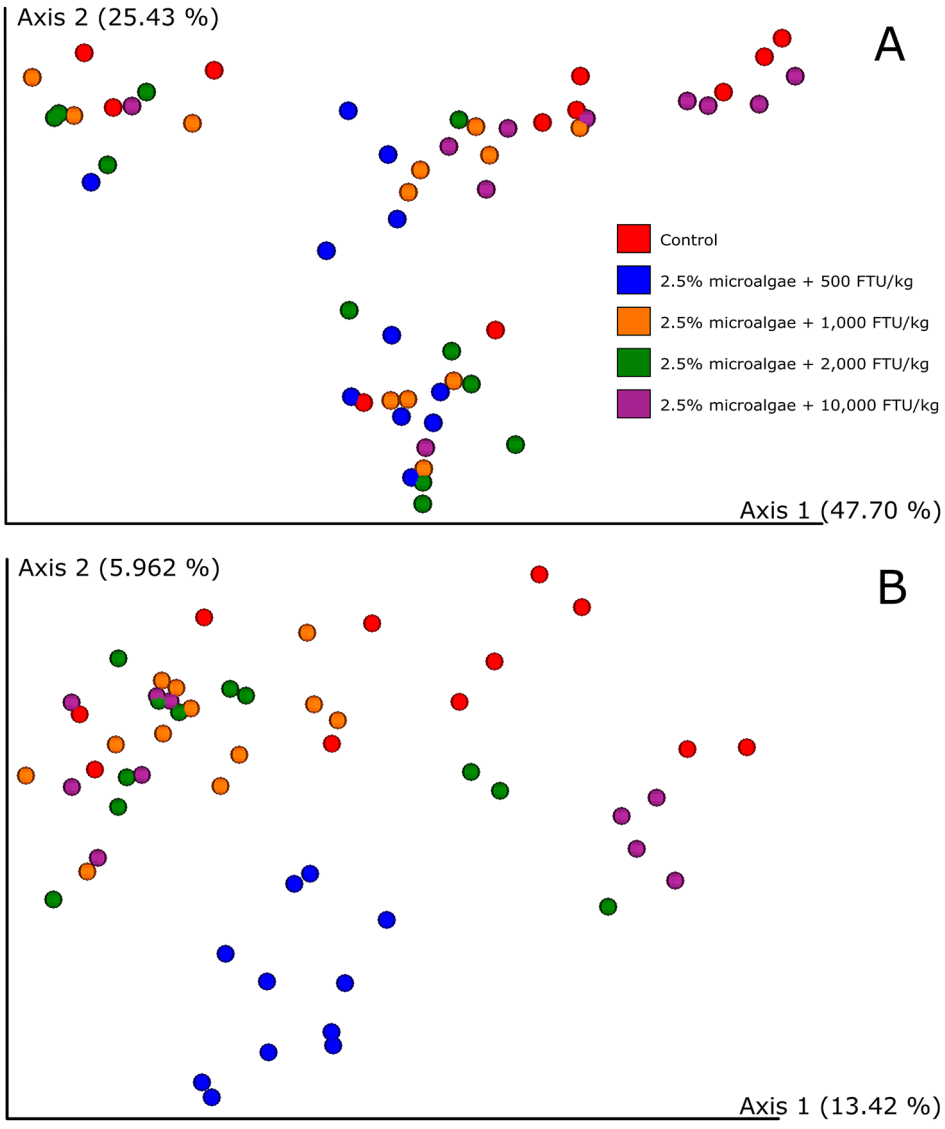

Fig. 4 Dimensional figures showing the first two axes of the Principal Coordinate Analysis and representing bacterial communities in the gut of juvenile European seabass (*Dicentrarchus labrax*) fed a control diet or a diet supplemented with 2.5% microalgae (*A. platensis* and *N. gaditana*) and different phytase concentrations (500, 1,000, 2,000, or 10,000 FTU/kg) using Weighted (A) and Unweighted UniFrac (B) distance matrices. Samples are color-marked by treatment (Control - red; 500 FTU/kg - blue; 1,000 FTU/kg – orange; 2,000 FTU/kg – green; 10,000 FTU/kg – purple). The proportion of variance explained by each PCo axis is indicated

and feeding efficiency compared with the group receiving an algae- and phytase-free diet. A recent study [55] also reported that dietary supplementation with 10,000 FTU/kg) improved the metabolism and intestinal functionality of European seabass juveniles. These results support the potential substitution of diets based on fishmeal, fish oil, or soybean meal with diets enriched with microalgae and phytase.

To our best knowledge, no previous study has examined the effect on gut microbiota of an aquaculture diet that combined a blend of crude microalgae (*A. platensis* and *N. gaditana*) with different concentrations of exogenous phytase. Dietary supplementation with microalgae but without phytase has been studied in various aquaculture fish species with contradictory results. Thus, a crude microalgae-supplemented diet reduced alpha diversity indices in gilthead seabream (*S. aurata*) [61], while a diet containing hydrolyzed *N. gaditana* produced no change in microbial diversity [62]. By contrast, total fishmeal replacement by *A. platensis* in African catfish (*Clarias gariepinus*) increased their alpha diversity [63]. These disparities may be attributable to differences between the fish species, microalgae doses, or methods used to prepare the microalgae biomass for inclusion in aquafeeds. A recent study [64] found that European seabass fed diets in which a blend of microalgae (*Nannochloropsis* spp.)

Table 4 Permutational ANOVA on the effects of diet and tank nested in diet on beta diversity indices of gut microbiota of European seabass juveniles fed the control diet or a diet supplemented with 2.5% microalgae blend (*A. platensis* and *N. gaditana*) and different phytase concentrations (500, 1,000, 2,000, or 10,000 FTU/kg). D.f. = degree of freedom, the first number being the d.f. of the independent variable and the second the d.f. of the error term. Significant *p*-values are indicated in bold

β-Diversity Distance Matrix	Explanatory variables	D.f.	Pseudo-F	*P*
Weighted UniFrac	Diet	4,39	4.88	**0.002**
	Tank (Diet)	10,39	0.56	0.976
Unweighted UniFrac	Diet	4,39	1.61	**0.001**
	Tank (Diet)	10,39	1.04	0.265

replaced fishmeal or fish oil had a higher bacterial alpha diversity compared with fish receiving a microalgae-free diet. Likewise, other authors described positive effects of microalgae-enriched diets on the bacterial community [65].

Recent studies in Nile tilapia (*O. niloticus*) observed no changes in gut microbial diversity when microalgae supplementation was combined with dietary phytase [66, 67] or a blend of exogenous phytase, protease, and xylanase [68]. In the present study of European seabream juveniles, the effect of phytase on the bacterial community in their gut depended on the concentration added to the feed. Thus, a significant decrease in Shannon's diversity index and a marginally significant decrease in Faith's phylogenetic diversity index were observed in the fish fed intermediate concentrations (1,000 or 2,000 FTU/kg). Alpha diversity values did not differ between fish fed the highest concentration (10,000 FTU/kg) and controls. According to these findings, the effect of including phytase in microalgae-supplemented diets may be dose dependent. While the gut microbiota of fish fed the lowest and highest doses of phytase (2,000 and 10,000 FTU/kg) showed a similar bacterial diversity to that of control fish, intermediate doses of phytase produced a reduction in diversity, involving majority ASVs of the intestinal microbial community. It has been reported that a high dose of phytase (10,000 FTU/Kg) is related to greater digestive enzyme activity and an improved intestinal morphology, with an increase in villi height and decrease in villi diameter [55]. It could therefore be hypothesized that diets with intermediate phytase concentrations might not allow the complete hydrolyzation of dietary phytate, which would affect the gut bacteria and/or intestinal morphology. By contrast, the complete phytate degradation obtained at a high phytase dose (10,000 FTU/kg) would avoid changes in gut morphology and would increase nutrient availability.

Conclusions

Supplementation of plant-based diets with a 2.5% crude microalgae blend (*A. platensis* and *N. gaditana*) plus 10,000 FTU/kg can be recommended for juvenile European seabass, because it improves their growth performance without affecting their gut microbial diversity. The effects on growth parameters and gut microbiota of this type of diet have not previously been examined in the same study. Further research is warranted to explore the effects on growth performance and gut microbiota of higher concentrations of microalgae with lower concentrations of fishmeal and/or fish oil.

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Author contributions

MMR, FJA-L, EV and MM-B designed the study including the experimental approach. JMP-S, FJA-L, MM-B and EV supervised the research. AJV, JAM-S, AB and SF-M conducted the experiments with fish. JM-V prepared microalgae cultures and diets. MRR, JMP-S and AMMP performed the data curation, sequence analysis, bioinformatics and statistics. MRR and JMP-S wrote the original draft of the manuscript. All authors contributed to the final version of the manuscript as well as all authors read and approved the final version of the manuscript.

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Data availability

Sequences are available in the Sequence Read Archive (SRA) in the Genbank - NCBI webpage (https://www.ncbi.nlm.nih.gov/sra/), BioProject: PRJNA749674, Accession Nos. SAMN20396460 to SAMN20396707. Data used in the analyses of this manuscript are available in doi:10.6084/m9.figshare.22140911.

Declarations

Competing interests

The authors declare no competing interests.

Conflict of interest

All authors declare no conflict of interest.

Ethics approval and consent to participate

This study was performed in accordance with ARRIVE guidelines (https://arriveguidelines.org). Fish were kept and handled following the guidelines for experimental procedures in animal research of the Ethics and Animal Welfare Committee of the University of Cadiz and in accordance with Spanish (RD53/2013) and European Union (2010/63/UE) legislation. The Ethical Committee of the regional Government approved the experiments (Junta de Andalucía, reference number 23/https://doi.org/10.2019/176). The study was performed in the Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, University of Cádiz, Cádiz, Spain; Operational Code REGA: ES11028000312; authorization number 23/https://doi.org/10.2019/176, Junta de Andalucía, Spain).

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