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Research Article

Effects of Phytase and Microalgae Supplementation on the Utilization of Aquafeeds for European Seabass (*Dicentrarchus labrax*) Formulated with a High Inclusion Level of Plant Protein

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Numerous studies have reported the disadvantages of using plant protein in aquafeeds owing to the presence of antinutritional factors. Particularly, phytate can be overcome by dietary supplementation with exogenous phytase. On the other hand, the use of microalgae has been proven to be a valuable strategy for enhancing the digestive functionality in fish fed diets with high plant protein content. It was hypothesized that the simultaneous inclusion of phytase with microalgae can be useful to improve growth performance and digestive functionality in fish fed diets with high content of plant protein. In this study, the effects of feeds supplemented with different levels of exogenous phytase (FTU) and 2.5% of a blend of *Arthrospira platensis* and *Nannochloropsis gaditana* were evaluated on growth performance, metabolism, and gut functionality in European seabass (*Dicentrarchus labrax*) juveniles. Fish were fed *ad libitum* for 83 days with five diets containing different enzyme ratios (FTU), in triplicate: (i) control (0 FTU); (ii) 500 FTU; (iii) 1,000 FTU; (iv) 2,000 FTU; and (v) 10,000 FTU. At the end of the feeding period, zootechnical indexes were estimated, and biological samples were withdrawn for physiological determinations. The results obtained showed an overall improvement in the productive efficiency, general metabolism, and intestinal functionality in fish fed the highest phytase inclusion (2,000 and 10,000 FTU). Final weight (75.51 ± 1.01 g vs. 66.76 ± 1.26 g, $p = 0.005$), SGR (1.15 ± 0.02 vs. 1.00 ± 0.02, $p = 0.006$). and feed efficiency ($0.80 \pm 0.02\%$ day^{-1} vs. $0.67 \pm 0.01\%$ day^{-1}, $p = 0.012$) were higher in fish fed 10,000 FTU. The chemical composition of muscle and plasma metabolites did not vary among dietary treatments, except for the high glucose and low glycogen content in the liver with 2,000 FTU group. Focusing on gut functionality, enzyme activities tended to be higher in fish fed diets supplemented with phytase and microalgae, and significant differences were found for trypsin, chymotrypsin, and alkaline phosphatase with 1,000, 2,000, and 10,000 FTU, and leucine aminopeptidase with 2,000 and 10,000 FTU ($p < 0.0001$). Analysis of intestinal morphology revealed that 2,000 and 10,000 FTU combined with microalgae increased villi height and decreased villi diameter and enterocyte height. The presence of microalgae at low phytase supplementation level seems to lack of influence on the different parameters evaluated whereas only increased dietary phytase levels impacted on growth, nutrient utilization, and intestinal functionality. In addition, faecal samples were obtained by stripping the final portion of the intestines, from which the phosphorus and nitrogen were quantified. Apparent digestibility coefficient of phosphorus with 2,000 FTU and 10,000 FTU were higher, 74.3% and 77.8%, respectively, compared to control (52.4%). The results revealed a decrease in the amount of P shed with the excreta from the animals supplemented with phytase, a fact that evidenced an increased intestinal absorption of this element. Overall, the results obtained pointed out that phytase can be used at 10,000, and even 2,000, FTU in combination with microalgae as an approach to reducing feed costs and boost fish growth and digestive functionality of fish, while minimizing the environmental impacts of juvenile European seabass farms.

1. Introduction

In recent years, the substitution of fishmeal and fish oil by alternative ingredients in commercial aquafeeds has increased due to the need to obtain cost-effective and sustainable feeds, as well as to curb environmental pollution, overexploitation of fishery resources, and the decline of pelagic communities [1–3]. There is plenty of research aimed at evaluating the feasibility of including different plant-origin resources in aquaculture feeds. Recently, Hemre et al. [4] highlighted the potential and limitations of such ingredients. Consequently, during the last decade, the composition of commercial aquafeeds has shifted toward the inclusion of considerable amounts of terrestrial plant protein in their formulation. However, when it comes to carnivorous fish, their inclusion in diets is limited due to unbalanced amino acid profile, deficiency in $n-3$ HUFAS, and the presence of antinutritional compounds such as phytate and nonstarch polysaccharides (NAP), among others, which can induce detrimental effects on enzymatic activity, as well as changes in the digestive system and intestinal physiology of cultivated fish [5, 6]. All these limitations should be overcome in order to obtain dietary balanced nutrient profiles for fulfilling the requirements of farmed fish.

Phytic acid is an organic acid found in many plants, especially in seeds. This compound contains P, but in a form that is not bioavailable to fish, so that it is not absorbed through the intestinal mucosa, and therefore, it is eliminated through the faeces [6]. Moreover, phytic acid has a strong complexing activity on several minerals of nutritional importance, such as calcium, magnesium, iron, and zinc, which become insoluble, precipitate, and cannot be absorbed in the intestine, thus reducing their bioavailability. It is also well-known that phytate inhibits proteolytic enzymes [7]. Phytate is widely distributed among plants, and concentrations up to 7% can be found in seeds, in which phytic phosphorous can account up to 80% of total P [7, 8].

In this regard, the use of phytase, enzyme which has a role in phytate degradation, in aquafeeds has a double positive effect, since (i) on the one hand, it significantly improves the utilization of phosphorus contained in plant feedstuffs, increasing its bioavailability and reducing its discharge into the aquatic environment [9], and (ii) on the other hand, this enzyme improves weight gain and phosphorus utilization, increases carcass protein content, better protein retention, and enhances immune response in fish [10, 11]. Both effects together are considered relevant from an environmental point of view, owing to the reduction in inorganic discharges from aquaculture facilities.

Healthy diets, that include ingredients with functional properties on the organism, have been pointed out as a very promising option for several years. In this context, nutraceuticals develop a fundamental role, promoting numerous benefits for the health and physiological state of the animals, and particularly counteracting the antinutritional effects caused by terrestrial plant-based feedstuffs on intestinal functionality [12, 13]. The use of microalgae as functional ingredients has been contemplated as a suitable strategy for improving the intestinal functionality and ultrastructure, an even for improving muscle coloration, oxidative status, and general condition of farmed fish. Several studies have reported the advantages of microalgae as feed additive for aquaculture [14]. From a nutritional point of view, microalgae are a natural source of protein, lipids, vitamins, and carotenoids [12, 15] and, overall, their utilization has beneficial effects for fish [16–18]. It has been reported that the dietary inclusion of microalgae increases muscle protein, triglycerides, and omega-3 content and improves a variety of physiological activities, the intestinal functionality, and carcass quality [19, 20]. Some of the microalgae most used in aquafeeds are *Tetraselmis* sp., *Schizochytrium* sp., *Chlorella* sp., *Arthrospira* sp., and *Nannochloropsis* sp. [21]. Namely, *Arthrospira platensis* and *Nannochloropsis gaditana* show low phytic acid content and stand out as functional ingredients for enhancing the digestive functionality in fish [22–24].

Therefore, this research is aimed at evaluating the synergistic effects of using (i) different phytase levels (500, 1,000, 2,000, and 10,000 FTU/kg feed) and (ii) supplementing those diets with 2.5% of a blend of *Arthrospira platensis* and *Nannochloropsis gaditana* on growth performance, proximal composition of fish, skin color, intermediary metabolism, and intestinal functionality in European seabass (*Dicentrarchus labrax*) juveniles. In addition, possible improvement in P and N bioavailability owing to phytase and microalgae inclusion in diets is assessed. To our knowledge, there are not available other studies that evaluate the effect of combining phytase with microalgae for feeding aquaculture fish.

2. Materials and Methods

2.1. Diets. Crude biomass of *Arthrospira platensis* was produced in a 2,000 L raceway reactor located inside a greenhouse at Biorizon Biotech facilities (Almería, Spain), while *Nannochloropsis gaditana* was obtained from tubular photobioreactors in a pilot plant (EU H2020 SABANA facilities) at the Universidad de Almeria (Spain). Raw microalgae biomasses (approx. 15% dry matter) were freeze-dried and stored at –20°C until further use. The proximal composition of *A. platensis* was 65% crude protein and 5% crude lipid, whereas *N. gaditana* meal contained 44.5% crude protein, and 17.7% crude lipid, all values on dry matter basis. Both microalgae showed a phytic acid content lower than 0.1 g/100 g biomass.

Five isolipidic and isoenergetic experimental diets (49% crude protein, 15% crude lipid) were designed and formulated by the Experimental Diet Service at the Universidad de Almeria (Spain). Ingredients, proximal composition, and fatty acid profile of the experimental diets are shown in Table 1. The four experimental diets (designated as 500F, 1,000F, 2,000F, and 10,000F diets) contained different levels of phytase supplementation (500, 1,000, 2,000, and 10,000 phytase units, FTU, respectively, per kg), according to previous studies [25], and 2.5% of a blend of *A. platensis* and *N. gaditana* (1 : 1). The level of dietary microalgae was based on previous studies carried out with these algal strains in other fish species [23, 24]. The phytase activity (FTU) is defined as the amount of enzyme that releases 1 μmol of

TABLE 1: Ingredient composition (g/kg dry matter), proximate composition (% dry matter, mean ± SD, $n = 3$), and fatty acid profile (% total fatty acids, mean ± SD, $n = 3$) of the experimental diets.

Ingredient composition	CT	500F	1,000F	2,000F	10,000F
LT94 fishmeal[1]	100.0	100.0	100.0	100.0	100.0
Squid meal[2]	20.0	20.0	20.0	20.0	20.0
CPSP90[3]	10.0	10.0	10.0	10.0	10.0
Krill meal[4]	20.0	20.0	20.0	20.0	20.0
Microalgae blend[5]	—	25.0	25.0	25.0	25.0
Wheat gluten[6]	80.0	80.0	80.0	80.0	80.0
Soybean protein concentrate[7]	330.0	330.0	330.0	330.0	330.0
Pea protein concentrate[8]	80.0	80.0	80.0	80.0	80.0
Sunflower seed meal[9]	125.0	125.0	125.0	125.0	125.0
Fish oil[10]	60.0	60.0	60.0	60.0	60.0
Soybean oil	60.0	60.0	60.0	60.0	60.0
Soybean lecithin[11]	10.0	10.0	10.0	10.0	10.0
Wheat meal[12]	34.5	9.5	9.5	9.5	9.5
Choline chloride[13]	5.0	5.0	5.0	5.0	5.0
Betaine[14]	5.0	5.0	5.0	5.0	5.0
Vitamin and mineral premix[15]	20.0	20.0	20.0	20.0	20.0
Vitamin C[16]	1.0	1.0	1.0	1.0	1.0
Lysine[17]	12.0	12.0	12.0	12.0	12.0
Methionine[18]	6.0	6.0	6.0	6.0	6.0
Tryptophan[18]	1.5	1.5	1.5	1.5	1.5
Guar gum[19]	20.0	20.0	20.0	20.0	20.0
Phytase (mL/kg)[20]	—	0.4	0.8	1.5	7.7
Crude protein	49.1 ± 0.4	49.4 ± 0.6	49.4 ± 0.2	49.7 ± 0.5	49.1 ± 0.1
Crude lipid	15.3 ± 0.9	16.1 ± 0.2	15.3 ± 0.6	15.1 ± 0.5	15.5 ± 0.7
Ash	11.0 ± 0.5	11.7 ± 0.2	11.1 ± 0.4	11.2 ± 0.3	11.0 ± 1.0
Total phosphorus (P)	0.99 ± 0.02	1.02 ± 0.04	1.00 ± 0.06	1.02 ± 0.03	1.01 ± 0.02
Phytate (P)	0.22 ± 0.03	0.21 ± 0.04	0.20 ± 0.03	0.2 ± 0.02	0.21 ± 0.01
Moisture	5.8 ± 1.0	5.0 ± 0.3	5.8 ± 0.8	5.7 ± 0.7	5.9 ± 0.5
14 : 0	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	1.5 ± 0.1
16 : 0	18.4 ± 1.7	19.7 ± 0.2	19.5 ± 0.1	19.9 ± 0.3	18.2 ± 0.1
18 : 0	4.4 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.3 ± 0.4	4.3 ± 0.1
18 : 1*n*9	23.8 ± 0.5	23.3 ± 0.4	23.2 ± 0.1	23.4 ± 0.2	23.7 ± 0.1
18 : 2*n*6	33.0 ± 0.7	32.1 ± 0.5	32.0 ± 0.2	32.3 ± 0.1	32.9 ± 0.2
18 : 3*n*3	1.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	1.3 ± 0.1
20 : 4*n*6 (ARA)	0.9 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.3
20 : 5*n*3 (EPA)	3.5 ± 0.1	3.4 ± 0.3	3.4 ± 0.3	3.5 ± 0.4	3.4 ± 0.2
26 : 6*n*3 (DHA)	9.1 ± 0.2	8.9 ± 0.6	8.8 ± 0.1	9.1 ± 0.2	9.0 ± 0.1

Dietary codes: CT, control diet without microalgae and phytase; 500F, diet containing 2.5% microalgae + 500 FTU; 1,000F, diet containing 2.5% microalgae + 1,000 FTU; 2,000F, diet containing 2.5% microalgae + 2,000 FTU; and 10,000F, diet containing 2.5% microalgae + 10,000 FTU. [1]In total, 69.4% crude protein, 12.3% crude lipid (Norsildemel, Bergen, Norway). [2,3,4]Purchased from Bacarel (UK). CPSP90 is enzymatically predigested fishmeal. [5]A 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]In total, 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, and 6% crude lipid (Lorca Nutrition, 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 provider (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 bisulfite), 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 sulfate), 900 mg; Fe (iron sulfate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulfate) 750 mg; Ca (calcium carbonate), 18.6%; (186,000 mg); KCl, 2.41%; (24,100 mg); NaCl, 4.0% (40,000 mg). [16,17,18]TECNOVIT, Spain. [19]EPSA, Spain. [20]ePhyt® from Global Feed S.L. (Tervalis Group, Huelva, Spain). 1,309.9 phytase activity units (FTU)/mL. One FTU is defined as the amount of enzyme that releases 1 μmol of inorganic phosphate from phytate per minute under the specified reaction conditions in the International Standard ISO30024.

inorganic phosphate from phytate per minute under the reaction conditions specified in the International Standard ISO 30024 : 2009. Additionally, a microalgae- and phytase-free diet was used as control (CT). The enzyme included in the diets was ePhyt®, produced by Global Feed S.L. (Tervalis Group, Huelva, Spain). This enzyme is a bacterial 3-phytase (EC 3.1.3.8.) from a strain of *Komagataella phaffii* and belonging to the additive category "zootechnical additives" and to the functional group "digestibility enhancers," authorized as an additive in animal nutrition, according to Regulation (EC) 1831/2003 (additive number 4a25). The 10,000 FTU level was recommended by the manufacturer.

The diets were supplemented with 1% chromium oxide (Cr_2O_3) as inert marker to estimate P and N digestibility. All ingredients were mixed in a 60 L mixer, ground with a hammer mill (UPZ 100, Hosokawa-Alpine, Augsburg, Germany) to 0.5 mm. The diets were extruded in a five-section twin-screw extruder (Evolum 25, Clextral, Firminy, France), fitted with 3 and 4 mm die holes. The pellets were dried after extrusion at 27°C using a drying chamber (Airfrio, Almería) and cooled at room temperature. The liquid phytase was dissolved in distilled water and further added to feed pellets by vacuum coating on the following day in a Pegasus PG-10VC LAB vacuum coater (Dinnissen, Sevenum, The Netherlands), whereas microalgae were incorporated as powder during the mixing of feed ingredients before extrusion.

2.2. Animals, Experimental Design, and Fish Sampling

2.2.1. Fish and Feeding Trial. European seabass (*D. labrax*) juveniles were provided by a commercial company (Cultivos Piscícolas de Barbate S.L., Cádiz, Spain) and located at the indoor wet laboratories in the *Servicios Centrales de Investigación en Cultivos Marinos* (SCI-CM, CASEM, University of Cádiz, Cádiz, Spain; Operational Code REGA ES11028000312). Fish ($n = 375$, average initial body weight 29.1 ± 0.2 g) were randomly distributed in 15 400 L tanks adjusted to a final volume of 300 L (25 fish per tank, ~2.5 kg of fish/m^3 initial stocking density) in an open system circuit. Animals were acclimated for 1 week to seawater (38% salinity), natural photoperiod (September–December, latitude (36°35′06″N; 06°13′48″W; Cádiz, Spain) and constant temperature (18–19°C) prior to starting with the feeding protocol. Water parameters were continuously monitored and recorded by the Pacific Monitoring and Control System (OxyGuard International A/S, Denmark). During this acclimation period, fish were fed with the control diet formulated for this experiment (CT, Table 1); this period was also used to evaluate the higher feeding activity to stablish the best feed intake along the complete feeding trial spread over two daily meals. Fish were kept and handled following the guidelines for experimental procedures in animal research according to the Spanish (Real Decreto 53/2013) and European Union (Directive 2010/63/UE) legislation (authorisation number 23/10/2019/176, Junta de Andalucía). During the experimental feeding period (83 days), fish were fed *ad libitum* 6 days per week in two daily meals (10 : 00 am and 17 : 00 pm). The water in all the tanks was oxygen-saturated (>90% O_2 saturation) with air stones. Water ammonia (<0.1 mg/L), nitrite (<0.2 mg/L), and nitrate (<50 mg/L) were determined daily at 9 : 00 am with commercial kits (SERA® GmbH, Heinsberg, Germany).

2.2.2. Sampling. At the end of the feeding trial, overnight fasted fish (six animals per tank, $n = 18$ fish per experimental group) were anesthetized with a lethal dose of 2-phenoxyetanol (Sigma–Aldrich, 1 mL/L of SW). Blood samples were drawn through heparinized syringes, and centrifuged (3,000× *g*, 20 min, 4°C) to collect the plasma fraction. Prior to tissue collection, fish were killed by cervical severing. Fish were immediately weighted and dissected, and the whole intestine and liver were removed and weighed. Liver and muscle samples were frozen in liquid nitrogen and stored at −80°C until biochemical analysis. The fish carcasses were freeze-dried and stored at −20°C for further analysis of muscle proximate composition. For digestive enzyme activity determinations, whole intestine samples, without pyloric caeca and perivisceral fat, from six fish per tank were randomly collected to obtain three enzymatic pools from each experimental tank. The whole intestine samples were homogenized in distilled water at 4°C (0.5 g/mL). Supernatants were obtained after centrifugation (11,200× *g*, 12 min, 4°C) and stored in aliquots at −20°C until further use. Finally, samples of the proximal intestinal region (0.5 cm-sections cut 2 cm after the pyloric caeca) of three specimens from each tank were collected for examination by light microscopy (LM), and scanning (SEM) electron microscopy as detailed further. Color parameters were determined on the right side of the anterior dorsal skin of fish, and then a portion of muscle tissue (5 g) was removed for lipid oxidation determinations (TBARS).

In addition, at the end of the feeding trial, the remaining fish were fed manually, for 2 weeks, until apparent satiation once daily at 10 : 00 am on the same diets but supplemented with 1% inert marker (Cr_2O_3), and then faecal material was collected once skip-a-day (9 : 00 am) by manual stripping of each fish. The faecal samples collected in each tank were pooled within a tank and kept frozen at −20°C. All frozen samples were freeze-dried for analyzing phosphorus, nitrogen, and chromium. Apparent digestibility coefficient (ADC) was calculated according to the following equation [26]:

$$\mathrm{ADC}\,(\%) = 100 \times (1 - [\mathrm{Cr\ in\ diet} \times \mathrm{nutrient\ in\ faeces}] / [\mathrm{Cr\ in\ faeces} \times \mathrm{nutrient\ in\ diet}]), \quad (1)$$

where Cr in diet and Cr in faeces are the chromium contents in diet and faeces, respectively, nutrient in diet and nutrient in faeces stand for phosphorus and nitrogen contents in experimental diets and faeces, respectively.

2.3. Growth Performance and Biometric Parameters. The following growth parameters were evaluated: (i) specific growth rate (SGR) = 100 × (ln final body weight − ln initial body weight)/days; (ii) weight gain (WG) = 100 × (body weigh increase/initial body weight); (iii) feed efficiency (FE) = weight gain/total feed intake; and (iv) condition factor (*K*) = 100 × (body weight)/fork length^3). The biometric index obtained was (i) hepatosomatic index (HSI) = 100 × (liver weight)/fish weight). Zootechnical parameters were used to

estimate the global balance of feed costs for producing 1 ton of farmed fish according to Perera et al. [13].

2.4. Chemical Composition and Fatty Acids Analysis. Proximate analysis (dry matter, ash, and total protein, $N \times 6.25$) of feed, carcass, and muscle (six samples per tank) were determined according to AOAC [27] procedures. Lipids were extracted following the method described by Folch et al. [28] using chloroform/methanol (2 : 1 v/v) as solvent, and total lipid content was calculated gravimetrically. Fatty acid (FA) profile was determined by gas chromatography following the method described in Rodríguez-Ruiz et al.'s [29] study, by means of a gas chromatograph (HEWLETT PACKARD, 4890 Series II, Hewlett Packard Company, Avondale, PA), using a modification of the direct transesterification method described by Lepage and Roy [30] that involves no prior separation of the lipid fraction.

The determination of total P content was performed by the molybdovanadate phosphate method [31] adapted to a microplate assay. The reaction mixture consisted of 50 μL sample (prepared at 50 mg/mL) and 100 μL molybdovanadate reagent, followed by 10 min incubation. Phosphate–molybdovanadate complexes were measured spectrophotometrically at 415 nm (Multiskan EX ELISA plate reader, Thermo Labsystems, Cheshire, WA, USA). Each sample was determined in duplicate. For calculation of concentrations, a standard curve was prepared with $[PO_4^{-3}]$ in the range of 0–100 μg/mL. Chromium in faeces and diets was determined by atomic absorption spectrophotometer (Perkin Elmer 3300, Boston, USA) after acid digestion of the samples with 1.5 N HNO_3 + 0.38% KCl.

2.5. Biochemical Parameters on Plasma and Liver. Plasma levels of glucose, lactate, and triglycerides were measured using commercial kits from SPINREACT (St. Esteve de Bas, Girona, Spain) adapted to 96-well microplates. Plasma total protein concentration was determined with a BCA Protein Assay Kit (PIERCE, Thermo Fisher Scientific, USA, #23225) using BSA as standard. All assays were performed using a POWERWAVE 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) and the KCJUNIOR data analysis software for MICROSOFT.

Frozen samples of liver were (six samples pert tank) homogenized before metabolite analysis. Homogenization (66 mg tissue/mL) was carried out with a high-performance dispersion instrument (T 25 digital Ultra-Turrax®). A deproteinization step was performed using a 0.024 N perchloric acid solution, followed by neutralization with 1 M potassium bicarbonate. Homogenates were centrifuged (30 min, 3,250× *g*, 4°C), and supernatants were divided into aliquots. Triglyceride, lactate, and glucose levels were determined spectrophotometrically with commercial kits (SPINREACT), as described above. Glycogen concentration in liver extracts was quantified according to Keppler and Deckler [32], where glucose obtained after glycogen breakdown with amyloglucosidase (SIGMA-ALDRICH A7420) was determined with a commercial kit (SPINREACT), as described above.

2.6. Digestive Enzymatic Activities of Intestinal Tissue. Trypsin and chymotrypsin activities were determined using 0.5 mM M-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA) and 0.2 mM N-succinyl- (Ala)2-Pro-Phe-Pnitroanilide (SAPNA) in 50 mM Tris-HCl, 10 mM $CaCl_2$ buffer, pH 8.5, according to Erlanger et al. [33] and DelMar et al. [34], respectively. Leucine aminopeptidase activity was quantified using buffered 2 mM L-leucine-p-nitroanilide (LpNa) in 100 mM Tris-HCl, pH 8.8 as substrate, according to Pfleiderer [35]. Alkaline phosphatase activity was determined using buffered p-nitrophenyl phosphate (pH 9.5) as substrate [36]. One activity unit (AU) of trypsin, chymotrypsin, and leucine aminopeptidase was defined as the amount of enzyme that released 1 μmol of p-nitroanilide (pNA) per minute from the respective specific substrates (an extinction coefficient of 8,800 M/cm at 405 nm). For alkaline phosphatase, 1 AU was established as the amount of enzyme that released 1 μg of nitrophenyl per min (an extinction coefficient of 17,800 M/cm at 405 nm). All the assays were performed in triplicate and the specific enzyme activity was expressed as AU/g tissue.

2.7. Histology and Morphometric Analysis of Intestinal Mucosa. According to standard histological techniques performed in fish tissue [20], intestinal samples were fixed for 24 hr in phosphate-buffered formalin (4% v/v, pH 7.2), dehydrated, and embedded in paraffin. Transverse sections (5 μm) were cut, slides were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus ix51, Olympus, Barcelona, Spain) equipped with a digital camera (CC12, Olympus Soft Imaging Solutions GmbH, Muenster, Germany). Images were analyzed with specific software (Image J, National Institutes of Health, USA). The diameter and height of the mucosal folds (VD, VH), the height of the enterocytes (EH), and the thickness of the *lamina propria* (LT) were measured from the intestinal samples (20 measurements per slide × 2 slides per fish).

The scanning electron microscopy (SEM) samples were processed as detailed in [20]. The intestinal samples were fixed for 24 hr in phosphate-buffered formalin (4% v/v, pH 7.2), washed with phosphate buffer, and dehydrated in graded ethanol. Then, the samples were dried in a critical point dryer (CDP 030 Critical Point Dryer, Leica Microsystems, Madrid, Spain) using absolute ethanol as the intermediate fluid and CO_2 as the transition fluid, then mounted on supports, fixed with graphite (PELCO Colloidal Graphite, Ted Pella Inc., Redding, CA, USA) and fold sputter-coated (SCD 005 Sputter Coater, Leica Microsystems). All the samples were screened via high-resolution field emission scanning electron microscopy (FESEM) (Carl Zeiss, Sigma 300 VP, Jena, Germany). All the digital images were analyzed with Image J (National Institutes of Health, USA) software and a morphometric analysis was performed to determine the enterocyte apical area (EA) [37].

2.8. Determinations of Color and Muscle Lipid Oxidation. Color was measured in triplicate on the skin by the L^*, a^*, and b^* system [38], using a Minolta Chroma meter CR400 (Minolta, Japan). The parameters brightness (L^*, on a 0–100-point scale from black to white), redness (a^*, estimates the position between red, positive values, and green, negative values), and yellowness (b^*, estimates the position between

yellow, positive values, and blue, negative values) were determined.

Lipid oxidation was estimated according to Buege and Aust [39] in muscle samples after homogenization of 0.5 g in 4 mL 50 mM NaH_2PO_4 0.1% (v/v) Triton X-100 solution. The mixture was centrifuged (10,000 *g*, 20 min, 4°C) and the supernatant was mixed (1 : 5 v/v) with thiobarbituric acid (TBA) reagent (0.375% w/v TBA, 15% w/v trichloroacetic acid, 0.01% w/v 2,6-dibutyl hydroxytoluene, and 0.25 N HCl). The mixture was heated (100°C) for 15 min, then centrifuged (3,600 *g*, 10 min, 4°C), and the absorbance of supernatants was measured spectrophotometrically at 535 nm. The amount of thiobarbituric acid was expressed as mg of malonyl dialdehyde (MDA) per kg of muscle after comparing it with the MDA standard.

2.9. Statistics. Data are presented as mean and standard deviation (SD). All data were checked for normal distribution and homogeneity of variances and normalized when appropriate. All the data obtained were analyzed by means of a one-way ANOVA analysis taking dietary supplementation levels as the main factor, considering a significance level of 5% ($p<0.05$). Tukey's multiple range test was also performed to show the magnitude of differences among means. Additionally, after one-way ANOVA evaluation WG, SGR, and FE data were submitted to a second-order polynomial model ($y=ax^2+bx+c$) to determine the optimal dietary level of phytase, whereas the results of digestive enzyme activities and histomorphological measurement of intestinal mucosa to a linear model. All statistical analyses were carried out using GraphPadPrism software for Windows.

3. Results

3.1. Growth Performance and Biometric Parameters. The results obtained evidenced a significant increase in final body weight (Figure 1(a)), feed efficiency (FE, Figure 1(b)), specific growth rate (SGR, Figure 1(c)), and weight gain (WG, Figure 1(d)) in specimens fed with the 10,000F diet, while condition factor (*K*) did not show differences among experimental groups (Figure 1(e)). It should be also noted an increase in hepatosomatic index (HSI) in fish fed the 2,000F diet (Figure 1(f)). Quadratic polynomial regressions indicated the optimum dietary levels in the range 7,000–8,000 FTU/kg for WG ($y=-5\times10^{-7}x^2+0.008x+132.56$, $R^2=0.965$), SGR ($y=-3\times10^{-9}x^2+4\times10^{-5}x+1.01$, $R^2=0.967$), and FE ($y=-1\times10^{-9}x^2+3\times10^{-5}x+0{,}68$, $R^2=0.977$), respectively.

3.2. Chemical Composition of Fish. No differences were observed for any of the parameters of the proximal analysis considered for carcass and muscle samples of juvenile seabass ($p>0.05$) (Table 2).

Table 3 shows the fatty acid profile of juvenile seabass muscle. Overall, except for slight differences in oleic, linoleic, and eicosapentaenoic acids, no significant effects on the fatty acid profile attributable to the dietary inclusion of microalgae and phytase were observed. Polyunsaturated fatty acids (PUFA) were the predominant fraction with values between 41.9% and 45.9%, followed by monounsaturated fatty acids (30.0%–31.4%) and saturated fatty acids (21.4%–22.2%), with no significant differences among the treatments tested.

3.3. Estimation of Phosphorus and Nitrogen Waste. Figure 2 shows the ADCs of phosphorus and protein for the different diets. A dose-dependent effect is observed in the case of phosphorus, since P absorption increases as the dietary inclusion of phytase soars, with the higher P absorption in 2,000F and 10,000F ($y=0.0021x+59.304$, $R^2=0.625$). The apparent protein digestibility values did not show significant differences among the different diets, although a slight increase was observed in the 2,000F.

3.4. Blood and Tissue Biochemistry. The results obtained for plasma biochemistry indicated that circulating levels of triglycerides (TAG), protein, glucose, and lactate did not differ significantly among the experimental treatments ($p>0.05$), although certain tendency to decrease with the higher dietary enzyme concentrations (2,000F and 10,000F) was observed in triglyceride and lactate (Figure 3). Regarding liver extracts, no differences were found in triglycerides ($p=0.479$), but they were observed in relation to glucose, with the 2,000F batch showing significantly higher levels of free glucose, and significantly lower levels of stored glycogen, than the control batch (Figure 4).

3.5. Intestinal Functionality. Pancreatic secreted enzymes (trypsin and chymotrypsin) as well as intestinal secreted enzymes (leucine aminopeptidase and alkaline phosphatase) showed high levels of activity as phytase supplementation increased, with fish fed with 10,000F showing the highest levels of activity (Table 4). Leucine aminopeptidase ($y=0.00005x+0.394$, $R^2=0.765$) and alkaline phosphatase ($y=0.0005x+3.269$, $R^2=0.990$) activities fitted to linear regression models, evidencing the higher phytase supplementation, the higher enzyme activity.

The visual analysis of the micrographs obtained by light microscopy revealed no signs of intestinal damage in either the fish fed the CT diet or any of the supplemented experimental feeds (Figure 5). At lower objective magnification (10x), all the treatments showed abundant intestinal villi, whereas at higher objective magnification (20x and 40x), the images confirmed a normal appearance of the intestinal mucosa, with all enterocyte nuclei lined up in the basal area. Morphometric analysis of these histological images (Table 5) revealed a significant increase in villus height (VH) with the highest phytase dietary inclusion ($p<0.0001$). An inversely proportional relationship was observed between phytase enzyme inclusion, villus diameter, VD ($y=-0.0011x+115.3$, $R^2=0.6745$), and *lamina propria* thickness, LT ($y=-0.0005x+15.1$, $R^2=0.616$), with the lowest values for these parameters in fish fed with 2,000F and 10,000F diets. *Supplementary 1* shows the apical structure of intestinal mucosa under scanning electron microscopy. Likewise, it was also observed that the apical area of enterocytes (*Supplementary 2*) was similar in all the treatments tested, except for 1,000 F, with significantly lower values than those obtained in the rest of the dietary treatments.

3.6. Skin Color and TBARs Analysis. The instrumental color analysis of the skin pigmentation of seabass fed the different

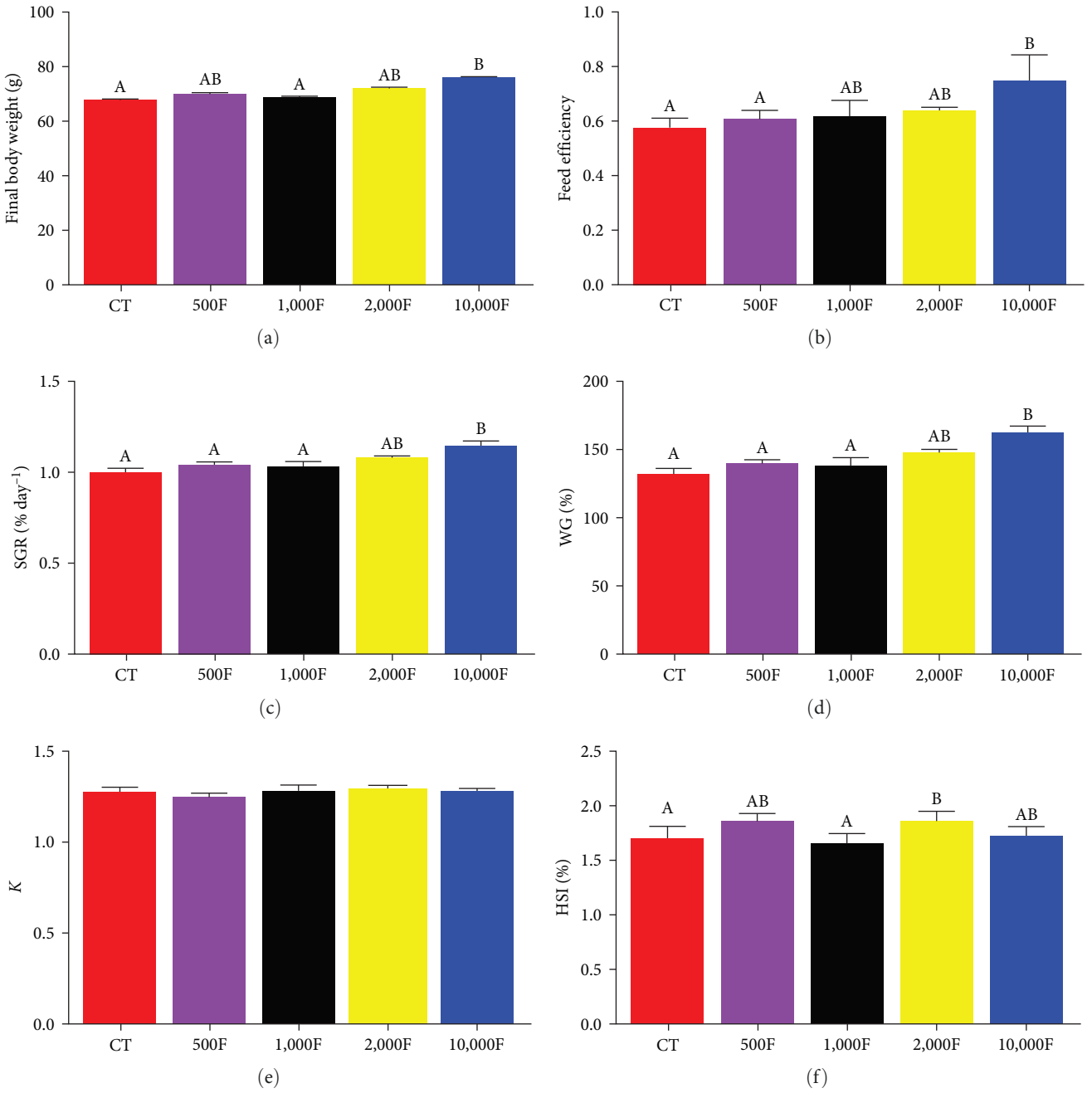

FIGURE 1: Biometric indices obtained in *D. labrax* specimens fed with different experimental diets. Final weight ((a) $p = 0.001$), feed efficiency (FE, (b) $p = 0.036$), specific growth rate (SGR, (c) $p = 0.006$), weight gain (WG, (d) $p = 0.005$), condition factor (K, (e) $p = 0.207$), and hepatosomatic index (HSI, (f) $p = 0.006$). Different letters indicate significant differences among dietary treatments ($p < 0.05$). Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F: diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

experimental diets showed that no differences were observed for the chromatic parameter L^*, but significant differences in parameters a^* and b^* could be detected. In the case of a^*, it was more negative in individuals fed the 2,000F diet and less negative in individuals fed the CT diet (*Supplementary 3*). On the other hand, b^* levels were higher in specimens fed the 500F and 2,000F diets, and lower in those animals fed the 1,000F and 10,000F diets. With regard to lipid oxidation, differences ($p = 0.007$) were observed in thiobarbituric acid-reactive substances (TBARS) measured in the muscle of juvenile seabass owing to dietary treatments, with the highest values for those animals fed the diet with the highest inclusion of phytase enzyme (10,000 F), and the lowest values for those fed with CT and 1,000F diets (*Supplementary 4*).

3.7. Overall Balance of Costs. The estimation of the potential economic benefits of supplementing fish with the experimental diets including phytase is shown in Table 6. Data indicate that

Table 2: Proximal composition (g/100 g DM) of carcass and muscle of juvenile seabass at the end of the feeding trial.

	CT	500F	1,000F	2,000F	10,000F	p
Carcass						
Protein	59.45 ± 0.07	58.65 ± 0.35	59.15 ± 0.35	58.2 ± 0.99	58.65 ± 0.35	0.277
Lipid	22.35 ± 2.62	21.7 ± 7.64	18.9 ± 8.06	14.45 ± 7.14	17.9 ± 5.23	0.749
Ash	13.65 ± 0.35	13.05 ± 0.21	13.15 ± 0.07	12.8 ± 0.99	12.75 ± 0.35	0.481
Muscle						
Protein	73.34 ± 0.93	72.44 ± 2.30	72.84 ± 2.61	74.72 ± 0.75	73.81 ± 2.03	0.773
Lipid	18.23 ± 0.94	19.04 ± 0.58	18.41 ± 0.66	18.63 ± 0.73	19.27 ± 1.22	0.573
Ash	5.47 ± 0.79	6.07 ± 1.13	5.88 ± 1.10	6.03 ± 0.89	5.79 ± 1.01	0.348

Values are expressed as mean ± SD ($n = 3$). Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

Table 3: Muscle fatty acid composition (fatty acids, %) of juvenile seabass fed the experimental diets.

	CT	500F	1,000F	2,000F	10,000F	p
14 : 0	1.51 ± 0.01^{b}	1.50 ± 0.01^{ab}	1.37 ± 0.05^{a}	1.48 ± 0.02^{ab}	1.42 ± 0.05^{ab}	0.036
16 : 0	15.61 ± 0.08	15.82 ± 0.02	15.42 ± 1.17	16.40 ± 0.15	16.08 ± 0.44	0.514
18 : 0	4.25 ± 0.01	4.27 ± 0.03	4.38 ± 0.36	4.35 ± 0.08	4.27 ± 0.05	0.911
16 : 1*n*7	3.03 ± 0.02	2.94 ± 0.01	1.95 ± 2.76	3.04 ± 0.03	2.94 ± 0.09	0.700
18 : 1*n*9	26.13 ± 0.07^{ab}	25.17 ± 0.10^{a}	27.51 ± 1.01^{b}	26.41 ± 0.01^{ab}	25.57 ± 0.58^{ab}	0.039
18 : 2*n*6	22.83 ± 0.08^{a}	24.49 ± 0.36^{ab}	26.05 ± 1.36^{b}	23.73 ± 0.17^{ab}	24.06 ± 0.55^{ab}	0.035
18 : 3*n*3	1.70 ± 0.04	1.62 ± 0.20	1.33 ± 0.04	1.50 ± 0.04	1.50 ± 0.03	0.066
20 : 4*n*6, ARA	0.92 ± 0.03	0.93 ± 0.04	0.94 ± 0.05	0.90 ± 0.02	0.92 ± 0.01	0.732
20 : 5*n*3, EPA	3.99 ± 0.03^{b}	3.84 ± 0.01^{ab}	3.92 ± 0.19^{ab}	3.85 ± 0.01^{ab}	3.55 ± 0.08^{a}	0.032
22 : 6*n*3, DHA	11.33 ± 0.06	11.45 ± 0.02	11.80 ± 0.66	11.21 ± 0.03	10.97 ± 0.23	0.238
SFA	21.37 ± 0.08	21.60 ± 0.06	21.75 ± 0.76	22.24 ± 0.08	21.77 ± 0.54	0.254
MUFA	31.28 ± 0.04	30.02 ± 0.11	31.35 ± 1.66	31.32 ± 0.08	30.27 ± 0.70	0.382
PUFA	42.37 ± 0.05	43.71 ± 0.17	45.89 ± 2.44	42.76 ± 0.58	41.94 ± 0.75	0.094
Others	5.31 ± 0.42	5.46 ± 0.79	4.48 ± 0.01	4.00 ± 0.69	5.76 ± 3.10	0.743
*n*3	18.61 ± 0.10	18.28 ± 0.16	18.90 ± 1.02	18.13 ± 0.74	16.95 ± 0.19	0.102
*n*6	23.76 ± 0.05^{a}	25.42 ± 0.33^{ab}	26.99 ± 1.42^{b}	24.63 ± 0.15^{ab}	24.99 ± 0.56^{ab}	0.038
*n*9	28.19 ± 0.10^{ab}	27.08 ± 0.12^{a}	29.40 ± 1.10^{b}	28.29 ± 0.05^{ab}	27.33 ± 0.61^{ab}	0.048
*n*3/*n*6	0.78 ± 0.01^{b}	0.72 ± 0.02^{ab}	0.70 ± 0.01^{a}	0.74 ± 0.03^{ab}	0.68 ± 0.01^{a}	0.011
EPA/DHA	0.35 ± 0.01^{c}	0.34 ± 0.01^{bc}	0.33 ± 0.00^{ab}	0.34 ± 0.01^{bc}	0.32 ± 0.00^{a}	0.007

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Values expressed as mean ± SD ($n = 3$). Values in the same row with different lowercase letters indicate significant differences among dietary treatments ($p < 0.05$). Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

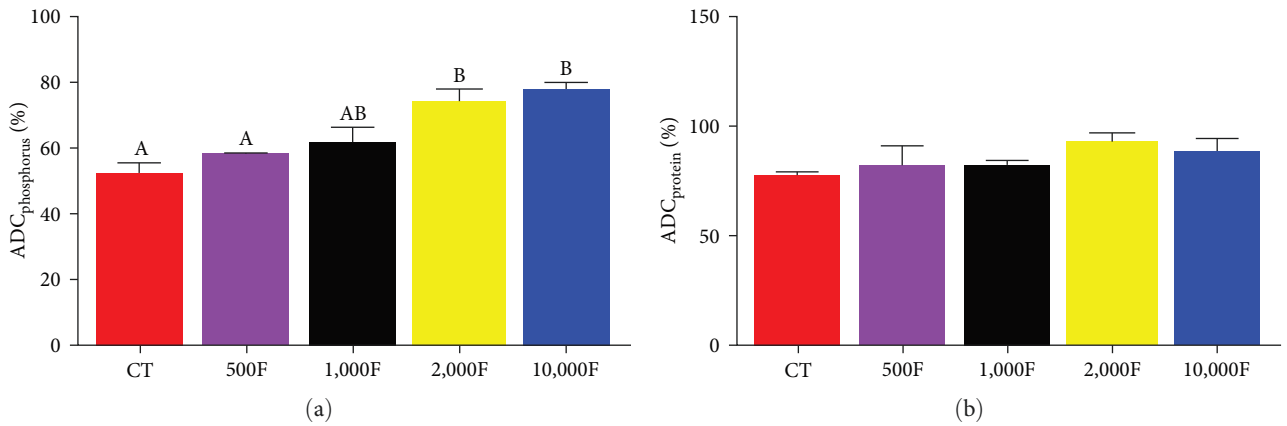

Figure 2: Apparent digestibility coefficients (ADC) of (a) phosphorus ($p = 0.002$) and (b) protein ($p = 0.353$) determined in fish fed diets supplemented with different levels of phytase (mean ± SD, $n = 3$). Values with different uppercase letters indicate significant differences among dietary treatments ($p < 0.05$). Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

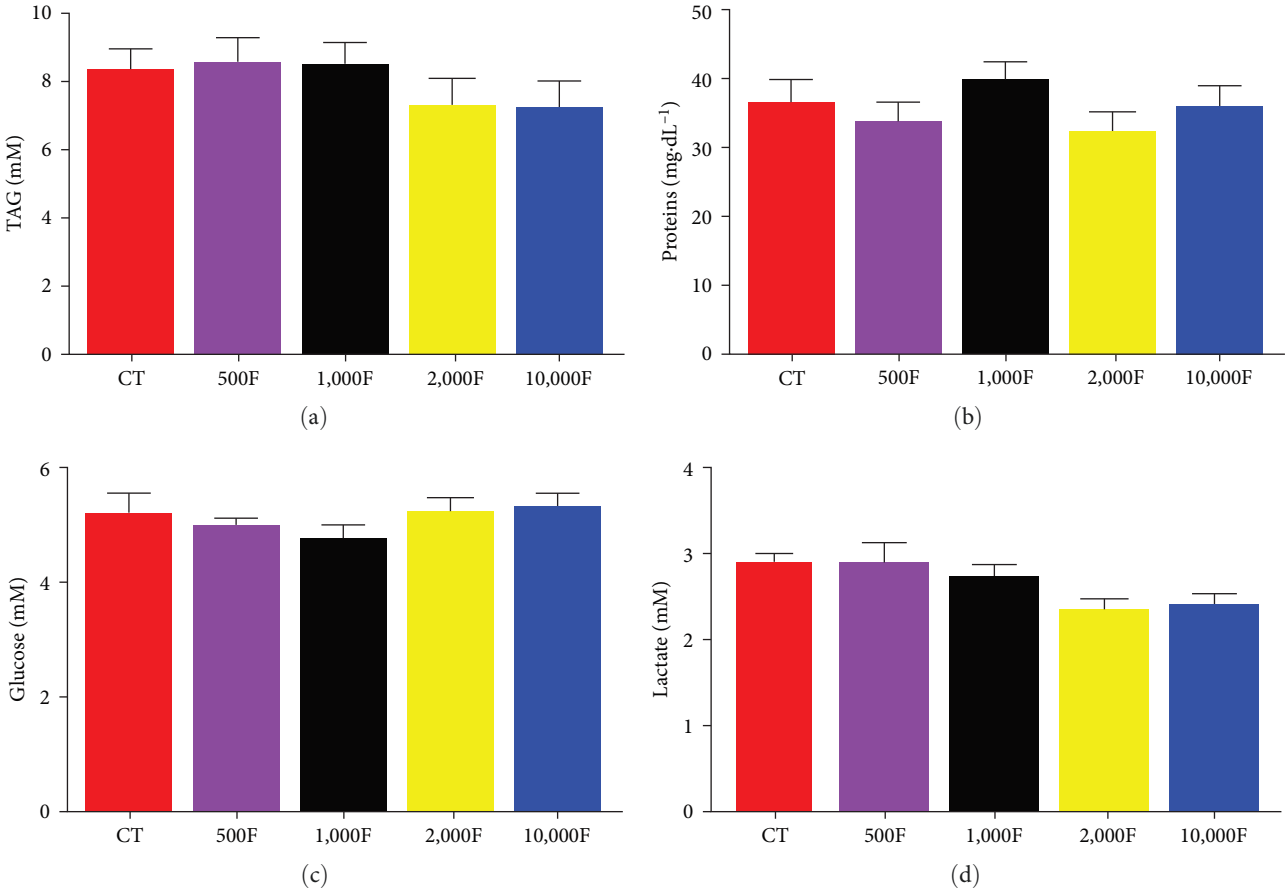

FIGURE 3: Results of plasma metabolites (a) triglycerides (TAG) ($p = 0.479$), (b) proteins ($p = 0.358$), (c) glucose ($p = 0.416$), and (d) lactate ($p = 0.229$) in seabass fed with different experimental diets (mean ± SD, $n = 6$). Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

1,000F, 2,000F, and 10,000F diets are more economically profitable than the 500F diet, since the overall balance of feed costs to produce 1 ton of farmed fish is lower compared to the CT diet, whose balance is higher.

4. Discussion

The objective of this study was to explore the complementary effect between phytase and microalgae dietary supplementation. With this aim, it was conducted the present study on juvenile European seabass (*D. labrax*), a carnivorous species, in which it was observed that feed efficiency increased significantly in specimens fed with the 10,000F diet compared to the rest of dietary treatments. The results obtained evidence that effect of phytase supplementation is greater than that promoted by the microalgae inclusion. This finding agrees with the initial hypothesis, since the highest level of phytase may provide the highest level of nutrient absorption and, therefore, the highest conversion ratios concomitantly with higher SGR found, as previously demonstrated in the same species by Kaushik et al. [40]. This implies not only higher feed utilization for the specimens fed the 10,000F diet, but also higher growth, according to the WG (weight gain) values. It is important to highlight that no significant differences in feed intake were observed among the experimental groups. Therefore, these results indicate that the dietary incorporation of this exogenous enzyme for *D. labrax* aquaculture can improve feed conversion ratio index, which ranged from 1.73 in CT to 1.34 in 10,000F in this trial. This might well represent a clear benefit for the profitability throughout the production cycle, related to the reduction of production costs. On the other hand, it also involves environmental implications, taking into account that increased plant protein in aquafeeds supposes a parallel reduction in the utilization of fishmeal and fish oil. In addition, individuals fed the 2,000F diet have a higher HSI than those fed the CT diet, suggesting the possibility of a greater capacity of energy reserves stored in liver to be used in possible events that may occur. In this group, growth performance tends to increase in comparison to fish fed the control diet, though not significant difference was evidenced, which support the fact that inclusion of microalgae has a limited role to improve the growth of animals.

Previous studies demonstrated that feed plays a key role in fillet quality, since variations in diet can be reflected in the proximal composition of the fish [41, 42]. Wan et al. [43]

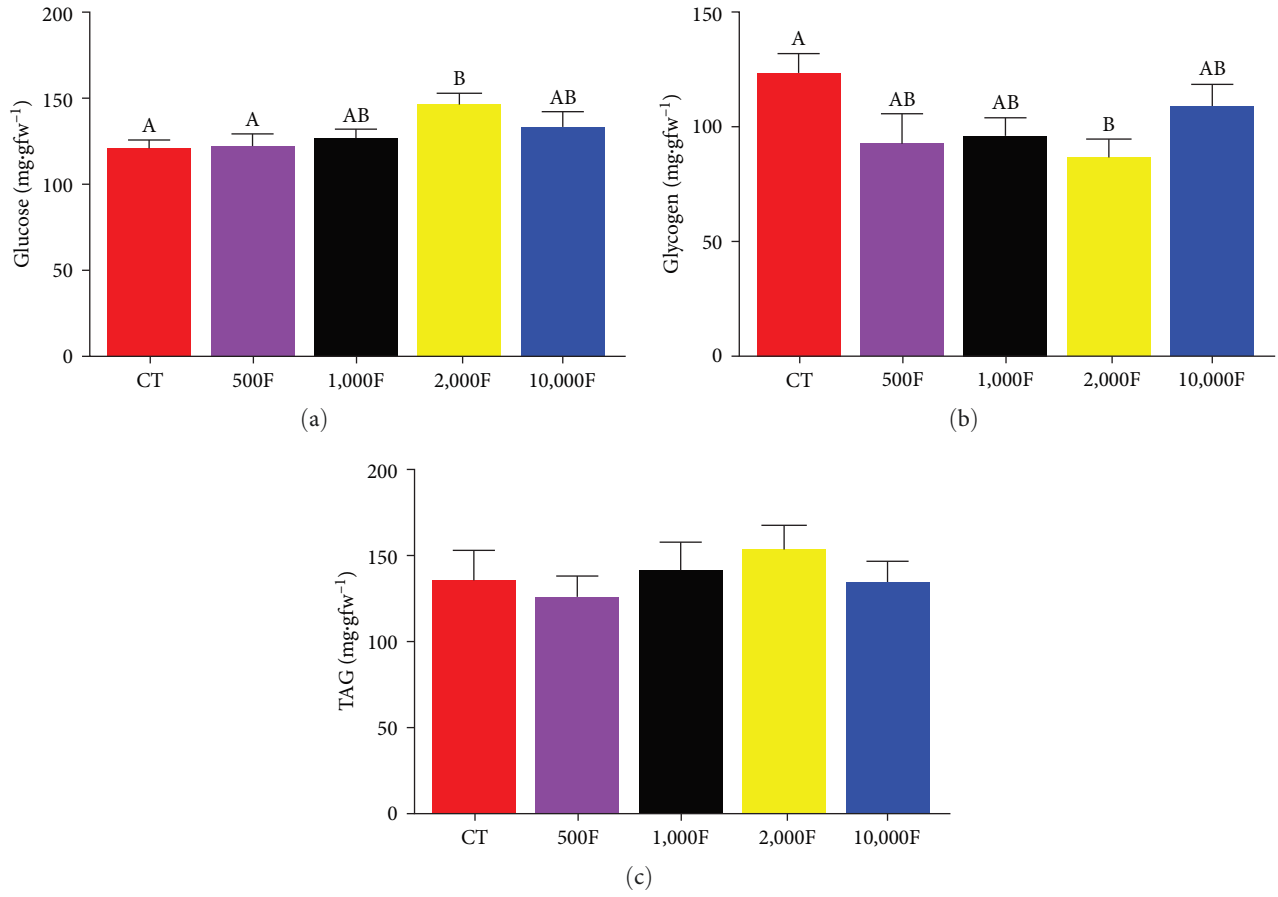

Figure 4: Results of liver metabolites measured in *D. labrax* specimens fed different experimental diets: (a) glucose ($p = 0.033$), (b) glycogen ($p = 0.049$), and (c) triglycerides (TAG) ($p = 0.707$). Different letters indicate statistically significant differences among dietary treatments (mean ± SD, $n = 6$). Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU. (gfw, g fresh weight).

Table 4: Digestive enzyme activities (AU/g tissue) measured in intestinal extracts of juvenile seabass.

	Trypsin	Chymotrypsin	Leucine aminopeptidase	Alkaline phosphatase
CT	0.04 ± 0.01^{a}	1.01 ± 0.17^{a}	0.34 ± 0.09^{a}	3.13 ± 0.35^{a}
500F	0.06 ± 0.02^{ab}	1.09 ± 0.24^{ab}	0.40 ± 0.05^{ab}	3.56 ± 0.29^{ab}
1,000F	0.08 ± 0.02^{bc}	1.37 ± 0.24^{c}	0.42 ± 0.05^{ab}	4.05 ± 0.29^{b}
2,000F	0.07 ± 0.01^{bc}	1.28 ± 0.14^{bc}	0.49 ± 0.11^{bc}	4.02 ± 0.39^{b}
10,000F	0.08 ± 0.01^{c}	1.34 ± 0.15^{bc}	0.56 ± 0.09^{c}	8.12 ± 0.64^{c}
p	<0.0001	<0.0001	<0.0001	<0.0001

Values expressed as mean ± SD ($n = 9$). Values in the same column with different lowercase letters indicate significant differences among dietary treatments ($p < 0.05$). Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU, 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

reported that dietary inclusion of algae (at a level lower than 10%) affects muscle proximal composition in *Nile tilapia* (*Oreochromis niloticus*), whereas Perera et al. [13] also demonstrated this fact when including low (1%) or very low (0.5%) levels of nutraceuticals compounds extracted from a blend of microalgae. However, in the present study, proximate composition of seabass was not modified by the dietary treatments. Similar results were obtained with the inclusion of 5% *N. gaditana* in juvenile turbot [44], Senegalese sole [45], and seabream [15, 23], as well as with the inclusion of 1,000 and 2,000 FTU in turbot [46] and seabass [47]. Other studies have shown separately that the substitution of fishmeal and fish oil by microalgae and the supplementation with phytase is beneficial for the performance of fish of different species and for the environment. For example, Phoco [48] and Roy and Pal [49] demonstrated the possibility of reducing fishmeal in aquafeeds up to 15% without affecting fish growth parameters and without altering the lipid and fatty acid profiles in tilapia (*Oreochromis mossambicus*). Dias and Santigosa [47] reported that the inclusion of

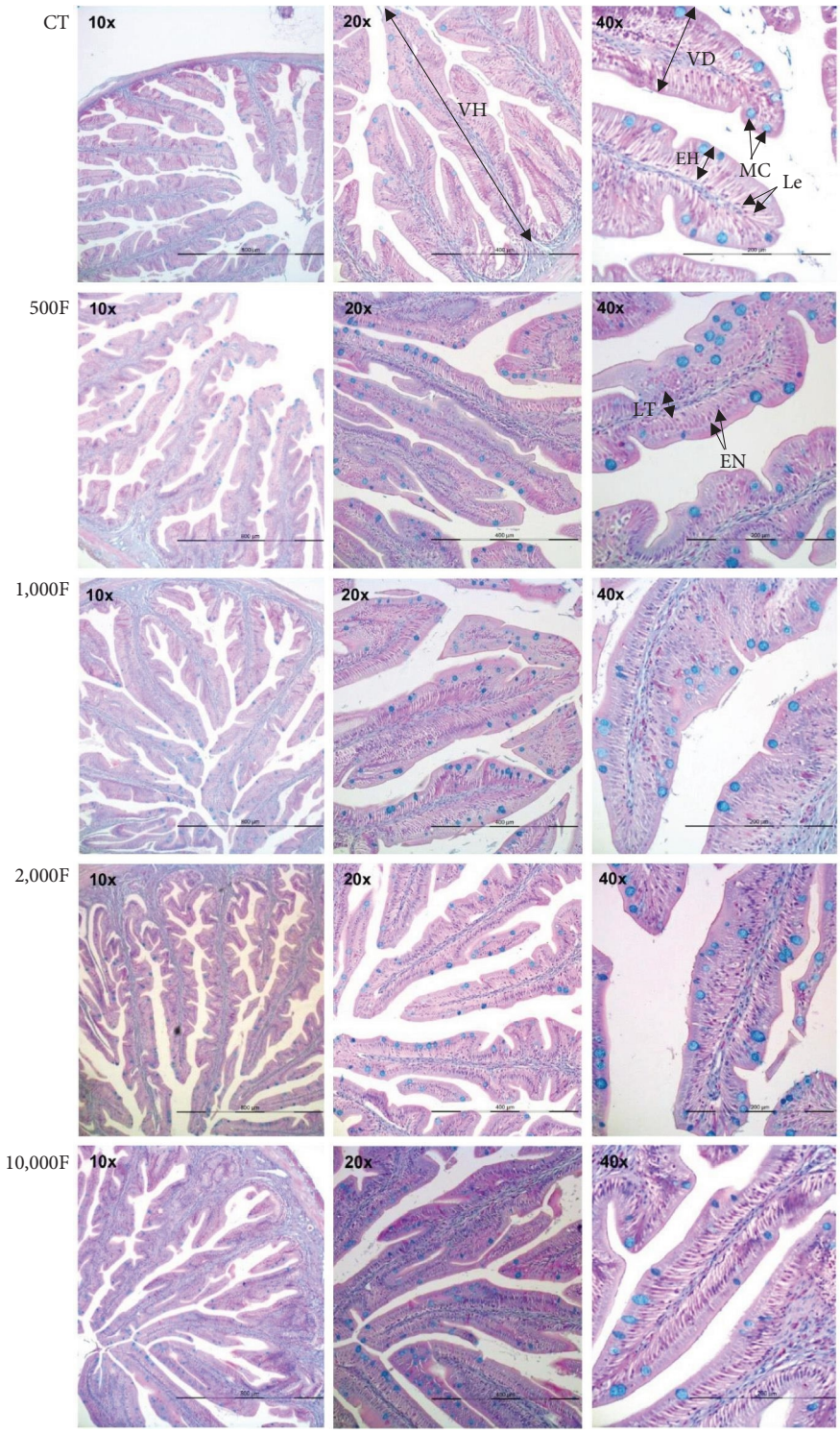

FIGURE 5: Details of the cross-section of the proximal intestines of juvenile seabass fed with the experimental diets. Objective magnification (H&E stain): 10x (scale bar: 800 μm), 20x (scale bar: 400 μm), and 40x (scale bar: 200 μm). VH, villus height; VD, villus diameter; EH, enterocyte height; LT, *lamina propria* thickness; MC, mucosa cell; Le, leucocytes; and EN, enterocyte nucleus. Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

Table 5: Quantification of histological parameters evaluated in the intestine of juvenile seabass fed with the experimental diets.

	VH (μm)	VD (μm)	EH (μm)	LT (μm)
CT	$1{,}066.23 \pm 92.53^{a}$	114.89 ± 8.04^{b}	46.26 ± 4.17^{b}	15.68 ± 2.99^{bc}
500F	$1{,}039.49 \pm 95.7^{a}$	115.64 ± 8.57^{b}	47.99 ± 3.27^{b}	13.52 ± 2.21^{b}
1,000F	$1{,}039.78 \pm 121.51^{a}$	117.94 ± 10.22^{b}	43.63 ± 2.90^{a}	16.50 ± 2.14^{c}
2,000F	$1{,}182.79 \pm 69.75^{b}$	108.53 ± 7.64^{a}	43.01 ± 4.14^{a}	12.32 ± 1.20^{b}
10,000F	$1{,}150 \pm 136.03^{b}$	105.32 ± 6.35^{a}	42.87 ± 4.27^{a}	10.58 ± 1.15^{a}
p	<0.0001	<0.0001	<0.0001	<0.0001

Values are expressed as mean ± SD ($n = 6$). Figures in the same column with different lowercase letters indicate significant differences among dietary treatments ($p < 0.05$). VH, villus height; VD, villus diameter; EH, enterocyte height; and LT, *lamina propria* thickness. Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU.

Table 6: Estimation of the economic benefits of the utilization of exogenous phytase in aquafeeds.

Diet	Feed efficiency (kg fish/kg feed)	Total feed (kg feed/mt fish)	Feed saving (kg feed/mt fish)	Cost feed saving (€/mt fish)	Phytase use* (L/mt feed)	Additive cost** (€/mt fish)	Balance cost saving (€/mt fish)***
CT	0.5767	1,595.7	—	—	—	—	—
500F	0.6060	1,600.0	−4.26	−6.39	0.382	18.34	−24.73
1,000F	0.6167	1,515.0	80.74	121.11	0.763	34.68	86.43
2,000F	0.6400	1,523.8	71.94	107.91	1.527	69.81	38.10
10,000F	0.7467	1,333.3	262.41	393.62	7.634	305.35	88.27

*Feed additive price: phytase (30 €/L). **The estimation includes only the cost of exogenous phytase supplementation (€/tn feed). ***Positive (>0) and negative (<0) values have lower and higher per mt fish produced than feeding animal with CT diet, respectively. Dietary codes: CT, control diet without phytase and microalgae; 500F, diet containing 500 FTU; 1,000F, diet containing 1,000 FTU; 2,000F, diet containing 2,000 FTU; and 10,000F, diet containing 10,000 FTU. The estimation it has been considered a value of 1.5 €/kg feed.

phytase at levels of 1,000 and 2,000 FTU can adjust the delicate balance among maximized animal performance, optimal welfare standards, and minimal environmental footprint. However, in the present study, the combination of phytase and microalgae did not modify the chemical composition of fish, and particularly, the diet 10,000 FTU had the potential to enhance seabass growth without causing detrimental impacts on its proximal composition.

It is well-known that marine microalgae species such as *Tetraselmis*, *Isochrysis*, and *Nannochloropsis* are rich in essential fatty acids and have proven to be beneficial as feed ingredients for marine organisms [50]. The blend of microalgae used in the present study contains *Nannochloropsis gaditana*, which is characterized by its ability to synthesize EPA. However, the low dietary inclusion (1.25%) did not increase the content of this fatty acid in the diets. That way the slight changes observed in the content of certain fatty acid, 18 : 1*n*9, 18 : 2*n*6, and 20 : 5*n*3, cannot be attributed to the phytase or microalgae supplementation. Several authors have reported that lipid peroxidation affects long-chain PUFAs [23, 51, 52]; however, in the present study, the different dietary treatments had not effect on this parameter, since low levels of lipid oxidation were measured in fish muscle (0.7 mg MDA/kg) in all the experimental groups, values that were within the range of perfect quality fish for human consumption [53]. In addition, skin coloration plays an important role in consumer acceptance of farmed fish, with intense skin coloration usually associated with high-quality products [54]. Skin pigmentation is influenced by a wide variety of factors such as genetic, environmental, physiological, dietary, and others [54, 55]. In the present study, significant differences were obtained in the parameter a^* in fish fed the 2,000F compared to control, though a general trend to show a greenish skin appeared in all the group fed diets supplemented with phytase and microalgae. From the consumer's perspective, those fish might be visually most attractive due to their coloration than control fish. As reported by Sáez et al. [23], it is likely that these results are mainly due to the incorporation of microalgae in the diet, which is the reason why the color differences did not appear in the individuals fed with the CT diet, which lacks 2.5% microalgae incorporated to the rest of diets. Further studies in commercial-size fish are requested for knowing the impact of these diets on fish quality attributes in long-term feeding assays.

The results showed that the incorporation of phytase tend to promote the utilization of dietary phosphorus, especially in fish fed the highest enzyme dose. The apparent digestibility results corroborated the initial hypothesis that diets with the higher inclusion level of exogenous enzymes (2,000F and 10,000F) improve the utilization of dietary phosphorus by enhancing the intestinal absorption, which ultimately reduces the amount of phosphorus excreted into the environment. These findings agree with those obtained by Günther et al. [56], who observed a 40% reduction of faecal phosphorus in tilapia juveniles after supplementing with 1,200 FTU. Extrapolated globally to the commercial production system, along with the improved assimilation and subsequent increase in growth observed particularly in fish fed the diet 10,000 F, these results suggest an environmental improvement. By reducing the levels of phosphorus released into the water through faeces, the phytase dietary supplementation

can contribute to mitigate environmental risks such as eutrophication.

Blood parameters are valuable indicators to show the physiological response to stress and general health conditions of fish against nutritional and environmental changes [57, 58]. In our study, the response of intermediary metabolism to the dietary treatments was assessed through its main energetic substrates both in plasma and liver. Glucose is the elementary unit of carbohydrates and in turn the basic bioenergetic unit for organisms. It is usually the first substrate to be affected by nutritional deficits [59]. In the present study, no significant changes in plasma parameters were observed, which indicates that none of the diets affected negatively the metabolic status of the animals, and that the all the feed supplemented with phytase and microalgae meet the minimum nutritional requirements for seabass, being able to produce the observed growth increase in fish fed the 10,000F diet. On the other hand, a significant increase was observed in the levels of hepatic glucose together with a decrease in glycogen in fish fed the 2,000F diet. This situation suggests that in this group, the incorporation of phytase and microalgae lead to glycogenolysis, which could be positive as the liver is one of the main metabolic reservoirs of energy, and glycogen level is considered a good indicator of the nutritional status of the animal [60]. For its part, TAGs are the elementary unit of lipids, represent the main source of lipid, and are an indispensable component of cell membranes [61]. TAGs usually increase their presence in plasma in response to stress, or to certain compounds in the diet [62], which implies a mobilization from the liver. In fact, the decrease in liver TAGs is a better indicator of a stressful situation that an increase in their content in plasma. In our study, despite not observing significant differences between the different dietary treatments, it can be observed a slight decrease in plasma TAG levels for the 2,000F and 10,000F groups, while, in hepatic TAG, an increasing trend for the same diets was evidenced. All this suggests that in these groups, the incorporation of phytase and microalgae may be causing these changes and improving the general status of the organisms [63]. Indeed, it has been reported that microalgae might well have certain impact on lipid turnover, mainly through the use of dietary lipid as energy source [64]. However, it is difficult to stablish the contribution of microalgae to the observed TAG variations. It can be hypothesized that in 2,000F and 10,000F groups, the higher digestive enzyme activity levels observed can increase the availability of certain intracellular algal compounds, which may activate lipid fish metabolism. However, further investigations are required to verify this hypothesis. Since in the present study, there were no differences in plasma total protein concentration among dietary treatments, it can be concluded that all experimental diets meet the basic requirements of seabass, and that any of the experimental diets did not influence nitrogen metabolism, as also demonstrated the content of nitrogen in faeces. Similar results were obtained by Dias and Santigosa [47], where the inclusion of phytase in the diet of seabream did not affect protein digestibility; however, the same study obtained dose-dependent results for tilapia, improving protein digestibility in the diet with the highest phytase inclusion. These discrepancies suggest that fish response could depend on various factors such dose-dependent effect and/or species-specific differences.

Phytate can bind digestive enzymes, thus reducing the bioavailability of various dietary components as well as fish performance. Yoo et al. [65] reported that phytate can interfere with the activity of digestive enzymes such as trypsin and chymotrypsin. The addition of phytase could hydrolyze phytate, thereby removing the inhibitory effect on digestive enzymes. Liu et al. [66] reported that the increase in digestive enzyme activity might be partly attributed to the release of minerals (Ca^{2+} and Mg^{2+}) bound to the phytate by phytase supplementation, this leading to significant activation of digestive proteases. However, no significant effect of the addition of phytase on the activity of proteases was evidenced in trout fed 2,500 FTU/kg [67] though the same authors [68] reported 14% reduction in the activity of rainbow trout trypsin in presence of phytate, but a lack of effect on chymotrypsin activity. In the present study, chymotrypsin and trypsin levels increased in 1,000F, 2,000F, and 10,000F groups, but not in 500F batch. This finding might indicate that (i) microalgae supplementation seems to contribute scarcely for increasing the digestive enzyme activities, and/or (ii) a specific threshold level of phytase supplementation (>500 FTU) is requested for achieving the observed effect. A similar response occurred in the case of leucine aminopeptidase and phosphatase alkaline activities, which might suggest a dose-dependent effect of phytase supplementation rather than the existence of an additional effect of the dietary use of microalgae. Indeed, a dose-dependent linear effect was observed in the latter two digestive enzyme activities. Namely, alkaline phosphatase is a dominant enzyme in the intestinal mucosa commonly used as an indicator of the intestinal integrity, and as a general marker of nutrient absorption. For this reason, an increase in this activity may be associated with an improvement in the overall efficiency of digestive and absorptive processes [69] giving rise, consequently, to the higher growth and higher P uptake observed in diets with higher levels of phytase. It would be expected that higher level of digestive enzymes might enhance nutrient utilization in those fish, but improvement in growth performance was evidenced only in 10,000F group. For this reason, a complementary mode of action between phytase and microalgae on digestive enzymes is not clearly demonstrated in this study, and further studies are needed for ascertaining this aspect by testing the 10,000F dietary treatment in absence of algal biomass.

The primary role of phytase as an additive is to increase the bioavailability and utilization of phytate-bound P; nevertheless, it might also provide new insights into the antinutritional properties of phytate. The intestine morphology parameters, such as VH, villus diameter (VD), enterocyte height (EH), and *lamina propria* thickness (LT) can be interpreted as indicatives of intestinal health since the proximal intestine is considered the main site of absorption in the fish gut. The intestine is the most important organ involved in the digestion and absorption of nutrients; hence, examination of this organ due to changes in the diet is of utmost relevance [70].

One of the main limitations for the use of plant proteins in aquafeeds is their impact on the digestive system, including reduced villi and EH, decreased brush border integrity, and supranuclear vacuolization in enterocytes, the presence of leukocytes in the *lamina propria* and submucosa, as well as the presence of different signs of inflammation, among others [71]. In the present study, histological examinations of intestine showed difference among the dietary groups. Fish fed on 2,000F and 10,000F evidenced longer villi, which might be used as indicators of greater capacity for digestion, absorption, and utilization of nutrients by the fish supplemented with the highest levels of phytase tested and 2.5% microalgae. Moita et al. [72] reported that phytase supplementation increased VH in birds, and this parameter has been correlated with greater nutrient absorption and utilization owing to the fact that longer villi may be an indicator of increased absorptive capacity and the existence of mature enterocytes [73, 74]. In the same sense, Ramos et al. [75] described that exogenous phytase supplementation (up to 1,696 FTU/kg) even at a low level (814 FTU/kg) reduced the intestinal damage caused by a soybean meal-based diet in *Mugil liza* juveniles. However, Ranjan et al. [76] found no changes in the intestinal structure in *Labeo rohita* fed diets supplemented with 500 FTU/kg. Those findings suggest that the effect of phytase are dose-dependent and species-specific, as evidenced the results obtained in the present study for the group fed the 500F diet. Moreover, fish fed 2,000F and 10,000F diets showed reduced villi diameter and *lamina propria* thickness, which reflects healthier intestinal epitheliums since an increase in both parameters is compatible with an enteritis-like effect. In contrast, Hu et al. [77] described that phytase supplementation can lead to inflammation and stress in the gut of tilapia, which might contribute to the failure of phytase (1,000 FTU/kg) to improve the overall growth performance of fish in their study. The microbial phytase used by those authors exerted disparate effects on tilapia in terms of gut health, improving microvilli density but reducing microvilli length. The authors proposed that the increased energy expenditure caused by gut inflammation might have counteracted the positive effects of dietary phytase on growth. In the present study, the apical surface of enterocytes (SEM images) had a normal appearance, and changes compatible with enteritis were not observed in any of the dietary groups, in spite of the fact that only the 10,000F group showed better growth performance compared to the control group. Regarding the role of microalgae in the abovementioned changes, it has been reported that algal biomass generated positive changes in the intestinal structure in marine fish (gilthead seabream and Senegalese sole) at different developmental stages, even at low dietary inclusion levels [20, 69, 78, 79]. However, in the present study, the lack of such effects in fish feed the 500F diet seems to indicate that the observed changes are more dependent on the exogenous phytase added rather than on the presence of microalgae, as it has been mentioned earlier in the case of digestive enzymes. High levels of dietary phytase increased the secretion of endogenous enzymes and resulted in longer villi, which promoted greater absorption of nutrients. But only in the case of 10,000 F, the higher phytase inclusion level promoted the higher growth performance observed.

5. Conclusions

Diets with a high percentage of phytase enzyme supplementation (10,000 FTU) led to overall improvements in growth performance, nutrient assimilation, utilization, and intestinal functionality, without adversely affecting any other biometric or physiological parameters. This resulted in a significant increase in the growth of fish fed the 10,000F diet. Metabolically, the dietary treatments yielded a homeostatic state in the plasma, although fish fed with 2,000F diet promoted the mobilization of liver glycogen, as indicated by increased plasma glucose levels in this experimental group. Considering the above findings, it can be concluded that 10,000F diet is the most suitable phytase inclusion level for juvenile seabass, but 2,000F could also be recommended as both are cost-effective, reduce P emissions to the environment, and promote intestinal functionality in juvenile seabass. The combination of phytase and microalgae can be a promising strategy to enhance feed efficiency and sustainability in seabass production, though further studies are needed for ascertaining the contribution of microalgae in phytase-enriched diets. It is crucial to continue researching to fully comprehend the mechanisms underlying these effects and their application in large-scale production systems.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary 1. Detail of the apical side of the enterocytes of the intestinal epithelium in juvenile seabass fed with the experimental diets. Scanning electron microscopy (SEM) images (SEM scale: 20 μm).

Supplementary 2. Morphometric analysis performed on SEM images from the intestinal mucosa of seabass specimens at the end of the feeding trial.

Supplementary 3. Effects of the different experimental diets on the chromatic parameters of seabass skin after the feeding trial (mean $\pm$ SD, $n = 12$). L^* ($p = 0.246$), a^* ($p = 0.046$), and b^* ($p = 0.0001$). Different letters indicate statistically significant differences among dietary treatments ($p < 0.05$).

Supplementary 4. Effects of the different experimental diets on the thiobarbituric Acid Reactive Substances (TBARS, mg MDA/kg) ($p = 0.007$) content in seabass muscle at the end of the feeding trial (mean $\pm$ SD, $n = 12$). Different letters indicate statistically significant differences among dietary treatments ($p < 0.05$).

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