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RESEARCH

High-solid loading alkaline autoclaved-processed *Codium tomentosum* as a sustainable feed ingredient for juvenile european seabass (*Dicentrarchus labrax*)

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Abstract

Codium tomentosum, a marine green macroalga is also produced in integrated multi-trophic aquaculture (IMTA) systems; however, its use in aquafeeds remains unexplored. This study evaluated the potential of *C. tomentosum* as a feedstuff for European seabass (*Dicentrarchus labrax*) and, for the first time, applied a high-solid-loading alkaline autoclave pre-treatment to enhance its nutritional value. Macroalgal biomass was treated with 1N NaOH (75% moisture) at 121 °C for 30 or 60 min, producing a slurry hydrolysate that was dried and incorporated at 7.5% into experimental diets. Four diets were tested in triplicate: a control diet, with no incorporation of *C. tomentosum*, a diet with untreated *C. tomentosum* (COD), and two diets including pre-treated *C. tomentosum* for 30 min (COD30) or 60 min (COD60). Two trials were performed: a digestibility trial (initial bodt weight (IBW) = 63 g) and an 11-week feeding trial with European seabass (IBW = 38 g) fed to apparent satiation. Inclusion of untreated *C. tomentosum* increased apparent digestibility but did not affect growth performance or feed efficiency. In contrast, fish fed the COD30 diet showed higher protein and amino acid digestibility and improved growth performance, feed utilization, and protein efficiency ratio compared with both control and untreated *C. tomentosum* diets. Extending the pre-treatment to 60 min did not lead to additional benefits and tended to reduce digestibility, feed intake, and growth. Whole-body composition and plasma biochemical parameters did not differ among treatments. Overall, a 30-min high solid-loading alkaline autoclave pre-treatment of *C. tomentosum* enhanced nutrient digestibility, growth, and feed utilization, supporting its potential as a sustainable ingredient in aquafeeds.

Keywords Chorophyceae · High-solid loading hydrolysis · Macroalgae processing · Digestibility · Sustainable feedstuff

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Introduction

Aquaculture is increasingly important for meeting the global need for seafood and other aquatic products. Growth in the sector is mainly driven by rising human consumption and greater market acceptance, and today, aquaculture provides over 50% of global fish production (Elsharkawy et al. 2022). Despite this progress, aquaculture still faces major challenges in terms of economic and environmental sustainability. The increase in feed prices threatens the economic viability of aquaculture, as feed production represents more than 50% of operating costs in intensive systems (FAO 2024). In addition, the reliance of feed formulation on a few ingredients, particularly fish meal and certain plant ingredients as soybean meal, increases pressure on wild fish stocks, agricultural land, and freshwater resources, thus increasing the environmental footprint of aquafeeds (Vizcaíno et al.

2016). For these reasons, research has focused on identifying suitable alternative ingredients that can improve both the economic and environmental sustainability of feed production (Glencross et al. 2007a; Tacon et al. 2009; Borquez et al. 2011).

Among potential alternatives, macroalgae have gained attention as a sustainable feed ingredients. They can be produced in enriched nutrient conditions, such as integrated multi-trophic aquaculture (IMTA) systems, which enhance their nutritional profile (Santizo-Taan et al. 2020). Macroalgae provide relevant amounts of nutrients and functional compounds, such as minerals, vitamins, antioxidants, and polyunsaturated fatty acids, which may help improve fish growth, immune status, antioxidant capacity, and stress response (Morais et al. 2020; Thépot et al. 2021). Several studies have shown that dietary inclusion of low levels of macroalgae (< 5%) may enhance fish health, resistance to infections, nutrient utilization, and carcass quality, depending on fish species, macroalgal species, and dietary inclusion level (Diler et al. 2007; El-Tawil 2010; Xu et al. 2011; Araújo et al. 2016; Vizcaíno et al. 2016, 2019; Shpigel et al. 2017; Sotoudeh and Jafari 2017; Lozano Muñoz et al. 2019; Magnoni et al. 2023). Further, economic analyses suggest that incorporating macroalgae in commercial fish diets may reduce feed costs (Saleh 2020).

The green macroalga, *Codium tomentosum*, is common in the eastern Atlantic Ocean from Europe to North Africa (Trowbridge et al. 2004; Valentão et al. 2010). It contains moderate levels of protein (21%) and lipids (5.8%) (Sousa et al. 2025), and is particularly rich in bioactive compounds with antioxidant, UV radiation protection, and anti-inflammatory properties (Martins et al. 2024). The production of *Codium* spp. in aquaculture is already established, with biomass being obtained through both extractive methods and production in IMTA systems (Marques et al. 2022). Although *C. tomentosum* has not yet been tested as a feed ingredient, extracts from this alga applied to fish fillets have been shown to improve their nutritional value, sensory quality, and antioxidant and antibacterial activity (Hafez et al. 2022).

Despite these advantages, the use of macroalgae as an ingredient for aquafeeds is limited by their low protein content and complex polysaccharides, which can hinder digestibility, particularly for carnivorous fish species (Bonvini et al. 2018; Fernandes et al. 2022). These polycarbohydrates tend to increase digesta viscosity and act as bulking agents, speeding up intestinal transit and reducing absorption (Bonvini et al. 2018). As a result, the overall digestive process may become less efficient, with potential implications for nutrient uptake and feed utilization (Kumar et al., 2012; Bonvini et al. 2018; Fernandes et al., 2019; Sartipiyarahmadi et al. 2023). To overcome this, various cell disruption techniques have been studied to reduce macroalgae-resistant polysaccharides, thereby enhancing their availability and simultaneously releasing nutrients trapped within the cellular matrix (Fernandes et al. 2022). The treatment selection, whether thermal, physical, chemical, or biological, depends on the type of macroalgae and the target nutritional and bioactive compounds (Kumar and Sharma 2017).

Alkaline treatments are known to promote cell wall degradation by altering the macrostructure and composition of macroalgal polysaccharides and converting the crystalline structure of lignocellulosic biomass into a more accessible form. Treatments, however, need to be optimized for each algal species (Francavilla et al. 2013; Pham et al. 2013). Although other treatments may be more selective, alkaline hydrolysis remains a relatively simple, cost-effective, and scalable method, ensuring its practical application (Pham et al. 2013). Conventional alkaline treatments are typically performed at low solid loadings, yielding two fractions: a liquid and a solid. This complicates downstream processing and requires large volumes of water. In contrast, high-solid loading alkaline treatments (higher than 15% of solids) with minimal water produce a single, slurry concentrated phase (Shafiei-Alavijeh et al. 2024). This strategy may improve the sustainability and cost-effectiveness of macroalgae processing by minimizing water requirements, simplifying downstream handling, and reducing material losses commonly associated with low-solid loading hydrolysis, which may indirectly contribute to lower energy demands (Shiva et al. 2022; Zhang et al. 2023).

Recent work has shown that applying a high-solid loading alkaline hydrolysis treatment to *C. tomentosum* reduces fiber content while increasing soluble protein and phenolic compounds (Ramos-Oliveira et al. 2025). Based on these results, the present study evaluated the potential of *C. tomentosum* as a feedstuff and assessed whether high-solid loading alkaline hydrolysis treatment could improve its bioavailability for European seabass (*Dicentrarchus labrax*), a commercially important marine fish widely produced in the Mediterranean region (Kousoulaki et al. 2015). The study evaluated the apparent digestibility, digestive enzyme activity, growth performance, whole-body composition, and plasma metabolites in juvenile European seabass fed diets containing *C. tomentosum*, either untreated or pre-treated using high-solid loading alkaline hydrolysis followed by autoclaving for 30 or 60 min.

Materials and methods

This study was approved by the ORBEA Animal Welfare Committee at CIIMAR (reference ORBEA_CIIMAR_27_2019), and all procedures complied with the ARRIVE guidelines and European and national legislation (European Directive 2010/63/EU; Portuguese Decree-Law 113/2013). Animal handling was performed by certified professionals in accordance with national legislation.

High solid loading alkaline autoclave treatment

Dried *Codium tomentosum* was mixed with 1 N NaOH (pH 11) in an amount sufficient to obtain a final moisture content of 75% (w/w). The treated biomass was then autoclaved at 121 °C for 30 min or 60 min, as described by Ramos-Oliveira et al. (2025). At the end of the hydrolysis process, the hydrolysed macroalgal biomass was dried for 48 h at 60 °C and milled to obtain a homogeneous powder with a particle size of 1 mm, and stored in sealed plastic bags at −4 °C until utilization.

Experimental diets

Four experimental diets were formulated to meet the nutritional requirements of the European seabass, with fishmeal and a mixture of plant feedstuffs as protein sources, and fish oil as a lipid source. The control diet was formulated without inclusion of *C. tomentosum*. Three other diets were formulated similarly but included 7.5% *C. tomentosum*, either untreated (COD diet) or treated by high-solid loading alkaline autoclave hydrolysis for 30 min (COD30 diet) or 60 min (COD60 diet), partially replacing wheat meal with minor adjustments in other ingredients to ensure that the diets remained isonitrogenous and isolipidic (Table 1). For the digestibility trial, the diets were supplemented with 0.5% chromic oxide (Cr_2O_3) as a digestibility marker. The dietary ingredients were thoroughly mixed and cold-pelleted with a 3-mm die using a laboratory pellet mill (California Pellet Mill, USA). Pellets were dried for 24 h at 60˚C, stored in plastic bags, and maintained at −4˚C until utilization.

Fish trials

The trials were conducted at CIIMAR, Matosinhos, Portugal, with juvenile European seabass (*Dicentrarchus labrax*) acquired from a commercial fish farm (Atlantik Fish, Algarve, Portugal). Upon arrival at CIIMAR, the fish were maintained in quarantine for 15 days before being transferred to the experimental facilities, where they were allowed to acclimate for more than 30 days.

Digestibility trial

The digestibility trial was performed in a recirculation aquaculture system (RAS) with twelve fiberglass tanks (60 L), each connected to a fecal settling column (Cho et al. 1982). At the start of the trial, six European seabass juveniles (average body weight of 63 g ± 0.5 g, mean ± SEM) were stocked in each tank, and the four experimental diets were randomly distributed among three replicate tanks per treatment. Fish were hand-fed to apparent satiety twice daily (8 am and 4 pm). After a 7-day diet adaptation period, feces were collected daily for 30 days. Before the morning feeding, feces settled in the columns were collected, centrifuged at 4,000 × *g* for 10 min, and then frozen until analysis. Feces from each tank were individually pooled to obtain sufficient material for analysis. To avoid contamination from uneaten feed, approximately 30 min after the second feeding, tanks, pipes, and settling columns were thoroughly cleaned to remove any residual feces and uneaten feed. The water parameters were maintained at the following levels: temperature, 21.4 ± 0.4˚C; salinity, 20 ± 2.0‰; pH, 7 ± 0.1; and dissolved oxygen, 7.8 ± 0.2 mg L^{-1}; total ammonia nitrogen < 0.05 mg L^{-1}, nitrite < 0.1 mg L^{-1}, and nitrate < 50 mg L^{-1}. The photoperiod was set at a 12-h light and 12-h dark cycle.

Chromium (Cr_2O_3) concentration in diets and feces was determined by acid digestion followed by spectrophotometric analysis, according to standard procedures. Apparent Digestibility Coefficients (ADCs) of dry matter, protein, amino acids, lipids, gross energy, and neutral detergent fiber of the experimental diets were calculated as follows:

$$ADC_{diet} = \left(1 - \left(\frac{dietary\ Cr_2O_3\ level \times feces\ nutrient\ or\ energy\ level}{feces\ Cr_2O_3\ level \times dietary\ nutrient\ or\ energy\ level}\right)\right) \times 100$$

Feeding trial

The feeding trial was conducted in a separate RAS system with twelve 500-L cylindrical fiberglass tanks supplied with filtered seawater and aerated through air stones. At the start of the experiment, groups of 16 European seabass (average body weight, 38 ± 0.0025 g, mean ± SEM) were randomly stocked into each tank, and the diets were assigned randomly in triplicate. The trial lasted 11 weeks, and the fish were hand-fed twice daily (8 am and 4 pm) for six days a week, until apparent satiation. Feed intake was recorded daily. Feed efficiency was calculated as total wet weight gain/total dry feed intake over the feeding trial. Water parameters were maintained at: temperature, 22.4 ± 1˚C; salinity, 20 ± 2.0 ‰; pH, 7.1 ± 0.2; dissolved oxygen, 7.8 ± 0.3 mg L^{-1}; total ammonia nitrogen < 0.05 mg L^{-1}; nitrite < 0.1 mg L^{-1}; and nitrate < 50 mg L^{-1}. The photoperiod was set at a 12-h light and 12-h dark cycle.

Table 1 Ingredients (% dry matter) and proximate composition (% dry matter) of the experimental diets used in the digestibility and feeding trials

	Ingredients			Diets			
	COD	COD30	COD60	Control	COD	COD30	COD60
Ingredients (% dry matter)							
Non-treated *C. tomentosum*[1]				—	7.5	—	—
30 min treated *C. tomentosum*[2]				—	—	7.5	
60 min treated *C. tomentosum*[2]				—	—	—	7.5
Fish meal[3]				20	20	20	20
Corn gluten[4]				12.5	12.5	12.5	12.5
Wheat gluten[5]				5.5	5.8	5.8	5.8
Soybean meal[6]				18.2	16.9	16.9	16.9
Pea protein concentrate meal[7]				10	10	10	10
Wheat meal[8]				15	8.1	8.1	8.1
Shrimp hydrolyzate[9]				1.2	1.2	1.2	1.2
Fish oil				13.7	13.9	13.9	13.9
Vitamin premix[10]				1	1	1	1
Choline chloride 50%[11]				0.5	0.5	0.5	0.5
Mineral premix[12]				1	1	1	1
Binder[13]				1	1	1	1
Dicalcium phosphate				—	0.2	0.2	0.2
Methionine				0.1	0.1	0.1	0.1
Taurine				0.3	0.3	0.3	0.3
Proximate analysis (% dry matter) and pH							
Dry matter	95.0	80.5	83.3	92.4	88.4	94.1	92.3
Protein	21.2	20.3	16.8	43.5	44.9	44.1	44.4
Lipid	1.1	1.1	1.1	16.4	17.0	17.8	16.9
Energy (kJ g^{-1})	13.3	13.0	12.9	23.6	24.3	21.2	23.4
Ash	36.5	51.2	57.2	5.8	8.8	10.7	9.4
Acid detergent fiber	6.4	4.2	2.5	3.44	3.89	2.96	3.56
Neutral detergent fiber	22.0	14.4	13.6	17.2	18.2	15.7	15.9
pH	6.06	9.87	9.83	5.82	5.54	6.26	6.29
Essential amino acids (% dry matter)							
Arginine, Arg				3.2	3.6	3.7	3.7
Histidine, His				1.8	1.7	2.0	1.5
Isoleucine, Ile				2.5	2.8	2.9	2.4
Leucine, Leu				11.7	12.7	11.9	10.8
Lysine, Lys				5.8	4.6	5.6	4.1
Methionine; Met				2.5	2.7	2.2	1.9
Phenylalanine, Phe				3.9	4.8	4.0	3.3
Threonine, Thr				3.3	2.4	2.7	3.4
Valine, Val				2.5	3.1	3.0	3.0
Non-essential amino acids (% dry matter)							
Tyrosine, Tyr				1.8	1.9	1.7	1.6
Alanine, Ala				11.5	12.1	11.2	10.7
Aspartic acid, Asp				6.0	5.4	6.2	5.4
Glutamic acid, Glu				10.9	8.9	9.5	10.2
Glycine, Gly				12.1	11.5	11.9	13.3
Serine, Ser				8.3	9.8	8.9	11.2
Proline, Pro				11.5	10.8	11.7	11.9

[1]AlgaPlus Lda. Aveiro, Portugal (CP: 21.2% CL: 1.1%)

[2]High-solid loading alkaline pre-treatment of *C. tomentosum* was performed using 1 N NaOH at a solid-to-liquid ratio of 25:75 (w/w), followed by autoclaving for either 30 min (COD30) or 60 min (COD60) (CP: 18.5% CL: 1.1%)

Table 1 (continued)

[3]Pesquera Centinela, Steam Dried LT, Chile (CP: 61.1%; CL: 9.8%). Sorgal, S.A., Ovar, Portugal

[4]Sorgal, S.A., Ovar, Portugal (CP: 75.4% CL:2.8%)

[5]Sorgal, S.A., Ovar, Portugal (CP: 54.9% CL: 2.2%)

[6]Non-GMO; Cargill France SAS, St. Germain-en-Laye, France (CP: 43.5%;CL:2.0%)

[7]Sorgal, S.A. Ovar, Portugal (CP: 49.4% CL: 3.3)

[8]Sorgal, S.A. Ovar, Portugal (CP: 13.2% CL: 2.4%)

[9]Sorgal, S.A., Ovar, Portugal

[10]Vitamins (mg kg^{-1} diet): retinol, 18,000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha-tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400

[11]Premix, Viana do Castelo, Portugal

[12]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet)

[13]Aquacube. Agil, UK

Sampling

At the end of the trial, after a 24-h fasting period, fish were lightly anesthetized (0.3 mL L^{-1} of 2-phenoxyethanol) and weighed. Body length was determined from digital images of lateral and dorsal views, according to Azevedo et al. (2023). Five fish from the initial stock and three fish per tank at the end of the trial were euthanized with an overdose of anesthetic (2-phenoxyethanol; 1 mL L^{-1}), followed by cervical section. Whole-body, liver, and visceral weights of each fish were recorded for hepatosomatic and visceral indices, and stored at −20ºC for whole-body composition analysis. Blood from three additional fish per tank was drawn from the caudal vein using heparinized syringes, centrifuged at 10,000 × *g* for 10 min, and the plasma was stored at −80 °C until analysis. Following blood collection, fish were euthanized and dissected on chilled trays. The digestive tract was excised, and the anterior, mid, and distal intestine samples were immediately frozen in liquid nitrogen and stored at 80ºC until analysis.

Proximate composition

Chemical analyses of feedstuffs, diets, whole-body samples, and feces were performed according to the AOAC (1980) methods. Dry matter was determined by drying the samples at 105˚C until constant weight; ash by incineration in a muffle furnace at 550˚C for 24 h; crude protein (N × 6.25) by acid digestion using Kjeltec digestion and destillator apparatus (Tecator Systems, Sweden, models 1015 and 1026, respectively); lipid by petroleum ether extraction using a SoxTec apparatus (Tecator Systems, extraction unit model 1043 and service unit model 1046); gross energy by direct combustion in an adiabatic bomb calorimeter (Parr model 6200; Parr Instruments, USA); ADF and NDF according to Van Soest et al. (1991) using a FIBRETHERM FT12 apparatus (13–0026, 12-place, automatic system).

Amino acid (AA) composition was determined following the protocol of the University of Vigo (Muñoz-Seijas et al. 2024). Samples were homogenized, hydrolyzed with 6 N HCl at 110 °C for 24 h, neutralized, and diluted in 0.1% formic acid. Quantification was performed by HPLC–MS/MS using a C18 column and multiple reaction monitoring detection. Cysteine and tryptophan were not determined. Dietary ingredients, proximate composition, and amino acid composition are presented in Table 1.

Digestive enzyme activity and plasma biochemical parameters

Intestine samples (n = 9 per diet) were homogenized using a Potter–Elvehjem homogenizer (dilution of 1:9 w/v) in an ice-cold 0,1 M phosphate buffer at pH 7.4. Homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C and the resulting supernatants were aliquoted and stored at −80 °C for further enzyme analysis.

All enzymatic assays were performed in 96-well microplates using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, China). Unless otherwise stated, assays were conducted at 37 °C, and each sample was analyzed in duplicate.

α-Amylase (EC 3.2.1.1) and lipase (EC 3.1.1.3) activities were determined using commercial assay kits (α-amylase-LQ, 1,004,102; and Lipase-LQ, 1,001,275; Spinreact S.A., Spain), according to the manufacturer's instructions, with adaptations for use in 96-well microplates. For each reaction, 10 µL of enzyme extract was mixed with 250 µL of the respective reagent solution. α-amylase activity was measured after 30 s of incubation by monitoring the formation of 2-chloro-4-nitrophenol at

405 nm. Lipase activity was assessed following 1 min of incubation by measuring methylresorufin production at 580 nm.

For total alkaline proteolytic activity, samples were incubated for 1 h at 37 °C (50 µL of enzyme extract mixed with 450 µL of reaction mixture) in a reaction mixture containing 1% (w/v) casein in 0.1 M Tris–HCl buffer, pH 8.0. The reaction was then stopped by adding 8% (w/v) trichloroacetic acid (500 µL). The mixture was kept at 4 °C for 1 h to facilitate protein precipitation, then centrifuged at 1,800 × *g* for 10 min. The absorbance of the supernatant was then measured at 280 nm using 200 µL transferred to a 96-well microplate. Tyrosine solution was used as a standard.

Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and total alkaline proteolytic activity were measured as described by Moutinho et al. (2017). Trypsin activity was measured using N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA, 0.5 mM) as substrate, and chymotrypsin activity was assessed using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA, 0.2 mM) as substrate. Reaction mixtures were prepared in Tris–HCl buffer containing calcium chloride (50 mM Tris–HCl and 20 mM $CaCl_2$, pH 8.5 for trypsin; 25 mM $CaCl_2$, pH 7.8 for chymotrypsin) and consisted of 20 µL of enzyme extract plus 200 µL of the respective substrate solution. The reactions were incubated at 37 °C for 10 min. The release of p-nitroaniline was monitored at 410 nm using its molar extinction coefficient (8.8 $mM^{-1} \cdot cm^{-1}$). Enzyme activity was normalized to the soluble protein content of the samples, which was quantified according to Bradford (1976), with bovine serum albumin as the standard.

Plasma biochemical parameters (total protein, albumin, glucose, cholesterol, and triglycerides) were measured in plasma samples from nine fish per dietary treatment (n = 9), using commercial Spinreact kits (refs.: 1,001,191, 1,001,091, 41,031, 1,001,291, and 1,001,023, respectively). For each assay, 5–10 µL of plasma was added to 200–250 µL of reagent according to the manufacturer's protocol, and absorbance was measured at the specified wavelengths using the same microplate reader.

Statistical analysis

Data are presented as mean and pooled standard error of the mean (SEM). All data were checked for normal distribution and homogeneity of variances. One-way ANOVAs were used to evaluate the different parameters and a probability level of 0.05 was used to reject the null hypothesis. The Tukey multiple range test was used to assess differences among means. All statistical analyses were performed using SPSS 25.0 for Windows.

Results

No differences among diets were observed in the apparent digestibility coefficients (ADCs) of lipids and NDF (Table 2). The ADC of dry matter was higher in the COD and COD30 diets than in the control, while the ADC of protein was higher in the COD30 diet compared with the control diet but did not differ from the other diets. The ADC of energy was higher in the COD diet than in the control diet. The ADCs of arginine, histidine, isoleucine, leucine, valine, and proline were also higher in the COD30 diet than in the control diet, but did not differ from those of the other diets. The ADC of lysine was higher in the COD30 diet than in the COD60 diet, while the ADC of methionine was higher in the COD diet than in the control diet. The ADC of phenylalanine was higher in the COD30 diet than in both the control and COD60 diets. Additionally, the ADC of serine was higher in the COD30 and COD60 diets than in the control diet.

During the growth trial, fish promptly accepted the experimental diets, and mortality was low and unaffected by diet composition (Table 3). Final body weight, weight gain, and daily growth index were higher in fish fed the COD30 diet than in those fed the control and COD60 diets, with no significant differences compared to fish fed the COD diet. Final body length was higher in fish fed the COD30 diet than in those fed the control and COD diets. Feed intake was higher in fish fed the COD30 diet than in those fed the COD60 diet, and feed efficiency was higher in fish fed the COD30 diet than in those fed the control and COD diets. The protein efficiency ratio was higher in fish fed the COD30 diet than in those fed the other diets. No significant differences were observed among diets in nitrogen or energy retention (% of the intake).

Whole-body dry matter, protein, lipid, and ash content were not affected by dietary composition (Table 4). The hepatosomatic index was significantly lower in fish fed the COD and COD60 diets compared to those fed the control diet. The viscerosomatic index was lower in fish fed the COD60 diet compared to the control diet.

Total alkaline protease activity was lower in fish fed the COD30 diet than in fish fed the control and COD diets (Table 5). In contrast, trypsin activity was higher in fish fed the COD30 diet than in those fed the COD and COD60 diets. The activities of chymotrypsin, α-amylase, and lipase did not differ among the dietary treatments.

Table 2 Apparent digestibility coefficients (%) of the diets

Diets	Control	COD	COD30	COD60	SEM	ANOVA *p*-value
Dry matter	66.8[a]	72.6[b]	75.5[b]	71.0[ab]	0.607	0.007
Protein	78.3[a]	85.2[ab]	88.5[b]	84.5[ab]	0.708	0.019
Lipids	80.2	86.1	84.5	82.0	0.435	0.482
Energy	71.6[a]	79.3[b]	78.9[ab]	77.2[ab]	0.589	0.041
Neutral detergent fiber	49.1	59.5	58.8	49.4	0.956	0.867
Essential amino acids						
Arginine, Arg	70.5[a]	81.4[ab]	88.6[b]	79.9[ab]	1.246	0.100
Histidine, His	86.2[a]	90.9[ab]	93.5[b]	87.5[ab]	0.550	0.020
Isoleucine, Ile	66.8[a]	75.9[ab]	83.4[b]	68.8[a]	1.254	0.024
Leucine, Leu	84.9[a]	89.9[ab]	92.9[b]	87.2[ab]	0.579	0.025
Lysine, Lys	93.9[ab]	94.0[ab]	95.8[b]	91.8[a]	0.276	0.041
Methionine, Met	91.7[a]	96.1[b]	95.3[ab]	92.7[ab]	0.353	0.021
Phenylalanine, Phe	77.1[a]	86.3[ab]	88.3[b]	77.0[a]	0.993	0.017
Threonine, Thr	70.2	69.3	80.4	75.1	0.853	0.189
Valine, Val	56.0[a]	69.1[ab]	78.8[b]	64.1[ab]	1.586	0.019
Non-essential amino acids						
Tyrosine, Tyr	70.5	83.0	84.9	78.7	1.071	0.053
Alanine, Ala	91.2	93.2	94.8	91.5	0.280	0.085
Aspartic acid, Asp	75.5	79.0	85.4	76.0	0.763	0.056
Glutamic acid, Glu	77.1	80.0	86.2	81.0	0.637	0.149
Glycine, Gly	93.4	94.0	95.8	93.1	0.200	0.105
Serine, Ser	84.2[a]	89.2[ab]	93.1[b]	90.1[b]	0.616	0.008
Proline, Pro	86.9[a]	89.0[ab]	93.8[b]	89.7[ab]	0.481	0.035

Results are presented as the mean (n = 3) ± standard error of the mean (SEM). Different letters in the same row indicate significant differences between diets (one-way ANOVA, $p < 0.05$)

Plasma levels of total protein, cholesterol, triglycerides, glucose, or albumin were not affected by the dietary treatments (Table 6).

Discussion

Assessing dietary digestibility and digestive physiology in diets containing novel ingredients is an important step in evaluating their nutritional potential, as it provides information on nutrient bioavailability and potential effects on digestive function. In the present study, the dietary replacement of wheat meal with untreated *C. tomentosum* increased the digestibility of dry matter and energy. However, protein and amino acid digestibility were further increased with the inclusion of *C. tomentosum* that had been autoclaved for 30 min under alkaline conditions (diet COD30). Protein digestibility increased by approximately 14%, while the digestibility of arginine, isoleucine, and valine increased approximately 30%; histidine, leucine, phenylalanine, and threonine increased approximately 10%; and lysine and methionine increased approximately 3% compared to the control diet. These results are in line with the previously reported increase in soluble protein content of *C. tomentosum* after high-solid loading alkaline autoclave treatment (Ramos-Oliveira et al. 2025), suggesting that autoclaving under alkaline conditions improves protein bioavailability.

Extending the autoclave treatment to 60 min did not lead to further improvement in protein digestibility and showed a tendency to reduce protein and amino acid digestibility (reductions of approximately 3 to 18% compared to COD30). This trend may be associated with heat-induced protein damage, such as protein cross-links formation or Maillard reaction products, depending on processing factors such as time, temperature, pH, and water activity (El Hosry et al. 2025). Several studies have demonstrated that optimizing heat processing conditions is essential to maximizing protein digestibility, as excessive heat or prolonged treatment may reduce the nutritional value of both animal and plant ingredients (Arndt et al. 1999; Sørensen et al. 2002; Hernández et al. 2010).

Although the differences were not statistically significant, the absolute values of energy and neutral detergent fiber digestibility were higher with the COD and COD30 diets compared to the control diet. However, despite a 21%

Table 3 Growth performance and feed utilization of European seabass juveniles fed the experimental diets

Diets	Control	COD	COD30	COD60	SEM	ANOVA *p*-value
Initial body weight (IBW, g)	38.0	38.0	39.0	38.0	0.002	0.355
Final body weight (FBW, g)	68.4^{a}	69.6^{ab}	82.0^{b}	67.6^{a}	1.133	0.019
Weight gain (g kg^{-1} day^{-1})	7.71^{a}	7.90^{ab}	9.90^{b}	7.55^{a}	0.183	0.025
Initial body length (IBL, cm)	16.0	16.1	15.9	16.1	0.160	0.413
Final body length (FBL, cm)	18.7^{a}	18.6^{a}	19.3^{b}	18.8^{ab}	0.743	0.013
Initial condition index [1]	0.93	0.91	0.95	0.92	0.002	0.425
Final condition index [1]	1.05	1.07	1.09	1.01	0.005	0.112
Daily growth index[2]	0.98^{a}	1.01^{ab}	1.33^{b}	0.96^{a}	0.029	0.021
Feed intake (g kg^{-1} day^{-1})	11.6^{ab}	11.6^{ab}	12.4^{b}	10.9^{a}	0.090	0.017
Feed efficiency[3]	0.67^{a}	0.68^{a}	0.81^{b}	0.69^{ab}	0.011	0.023
Protein efficiency ratio[4]	1.53^{a}	1.52^{a}	1.84^{b}	1.53^{a}	0.049	0.017
Nitrogen retention (% NI)[5]	23.7	23.6	29.5	23.6	0.686	0.200
Eenergy retention (% EI)[5]	33.2	36.7	38.1	36.0	0.912	0.789
Survival (%)	93.8	97.9	97.9	87.5	0.820	0.109

Results are presented as the mean (n = 3) and standard error of the mean (SEM). Different letters in the same row indicate significant differences between diets (one-way ANOVA, $p < 0.05$)

[1]Initial or final condition index = (IBW or FWB/IBL^3) × 100

[2]Daily growth index = (FBW$^{1/3}$ − IBW$^{1/3}$)/time in days) × 100

[3]Feed efficiency = wet weight gain/dry feed intake

[4]Protein efficiency ratio = wet weight gain/crude protein intake

[5]N retention (% N intake) = ((FBW × FBN content)—(IBW × IBN content))/(N intake) × 100

[6]E retention (% Energy intake) = ((Final carcass energy content—Initial energy content)/energy intake); ABW: Average bodyweight

Table 4 Whole-body composition (% fresh weight), hepatosomatic, and visceral indexes of European seabass juveniles fed the experimental diets

Diets	Initial	Control	COD	COD30	COD60	SEM	ANOVA *p*-value
Dry matter	26.9	35.1	33.3	33.9	34.3	0.397	0.871
Protein	15.0	15.2	16.1	16.3	16.1	0.175	0.598
Lipid	5.07	14.0	12.0	13.1	13.8	0.433	0.833
Ash	6.25	4.32	4.90	5.04	4.71	0.121	0.450
Gross energy (kJ g^{-1})	6.91	9.66	9.70	8.55	9.30	0.142	0.352
Hepatosomatic index[1]	-	2.22^{b}	1.46^{a}	1.85^{ab}	1.47^{a}	0.060	0.003
Viscerasomatic index[2]	-	11.3^{b}	9.34^{ab}	9.64^{ab}	8.61^{a}	0.190	0.045

Results are presented as the mean (n = 3) ± standard error of the mean (SEM). Different letters in the same row indicate significant differences between diets (one-way ANOVA, $p < 0.05$)

[1] Hepatosomatic index = (liver weight/whole body weight) × 100

[2] Viscerasomatic index = (visceral weight/whole body weight) × 100

Table 5 Digestive enzyme activity (mU mg^{-1} protein) of European seabass juveniles fed the experimental diets

Diets	Control	COD	COD30	COD60	SEM	ANOVA *p*-value
Total alkaline protease	131.3^{b}	128.1^{b}	46.4^{a}	77.3^{ab}	9.777	0.007
Trypsin	59.5^{bc}	45.4^{ab}	78.8^{c}	39.9^{a}	3.549	< 0.001
Chymotrypsin	3.81	3.91	4.03	4.18	0.177	0.928
α-amylase	14.0	18.1	20.4	22.4	1.348	0.592
Lipase	29.3	36.6	45.8	48.9	2.028	0.072

Results are presented as the mean (n = 9) ± standard error of the mean (SEM). Different letters in the same row indicate significant differences between diets (one-way ANOVA, $p < 0.05$)

Table 6 Plasma metabolite quantification (mg dL^{-1}) of European seabass juveniles fed the experimental diets

Diets	Control	COD	COD30	COD60	SEM	ANOVA *p*-value
Total protein	159.5	136.4	142.7	129.1	2.157	0.130
Cholesterol	122.7	123.1	129.6	136.6	1.094	0.348
Triglycerides	697.2	556.4	610.3	597.8	9.867	0.449
Glucose	64.4	67.4	63.4	63.2	0.323	0.928
Albumin	0.78	0.63	0.81	0.55	0.021	0.131

Results are presented as the mean (n = 9) ± standard error of the mean (SEM). The absence of different superscript letters indicates no significant differences (one-way ANOVA, $p < 0.05$)

reduction in neutral detergent fiber content of *C. tomentosum* after 30 min of alkaline autoclave hydrolysis, fiber digestibility remained similar in the COD and COD30 diets, likely due to the low dietary inclusion level (7.5% dry matter basis) of *C. tomentosum*. Similar observations were reported in Atlantic salmon (*Salmo salar*) fed diets containing either unfermented or fermented sugar kelp, where fiber digestibility remained unchanged despite variations in dietary fiber content (Sartipiyarahmadi et al. 2023). In European seabass, the physical and enzymatic treatment of macroalgae (*Ulva rigida* or *Gracilaria gracilis*) either reduced or did not impair the digestibility of protein, lipids, energy, or amino acids (Batista et al. 2020). Similarly, in rainbow trout (*Oncorhynchus mykiss*), the dietary incorporation of 5% and 10% autoclaved (121 °C for 30 min) *Ulva* spp. reduced protein digestibility, while lipid digestibility was unaffected, compared to the non-autoclaved *Ulva* meal (Güroy et al. 2013). In contrast, in vitro studies using digestive enzymes from *Chelon labrosus* demonstrated that sequential application of enzymatic hydrolysis followed by microbial fermentation of algal blends (*Tisochrysis* sp. and *Ulva* sp.) increased amino acid release after digestion (García-Márquez et al. 2025).

Macroalgae are characterized by high ash content, which may derive from intrinsic mineral accumulation and from associated inorganic materials such as salts or siliceous particles (Liu 2017). This was reflected in the higher dietary ash levels observed in diets incorporating *C. tomentosum*, an effect that was even more pronounced in diets containing the pre-treated biomass. In the present study, *C. tomentosum* was subjected to high-solids alkaline autoclave hydrolysis using NaOH, and the resulting hydrolysed biomass was incorporated into the diet without a prior washing step. Under these conditions, some sodium and other inorganic components from the NaOH solution may have remained associated with the hydrolyzed biomass, contributing to the higher ash content of the pre-treated material, as also reported previously (Ramos-Oliveira et al. 2026). Despite this, the present results indicate that the higher ash content did not impair nutrient utilization, as the COD30 diet showed improved protein and amino acid digestibility and no adverse effects on overall digestibility parameters at the tested inclusion level. This suggests that, under the present formulation conditions, the inorganic fraction did not represent a nutritional constraint. Thus, these findings suggest that the effects of dietary macroalgae inclusion on nutrient digestibility depend on the processing method and the inclusion level.

The digestibility of nutrients is closely linked to the activity of digestive enzymes (Silva et al. 2010), which, in turn, are modulated by the composition of the diet (Bakke et al. 2010). In the present study, no statistical differences were observed among diets in the activities of α-amylase, lipase, and chymotrypsin. Trypsin activity, however, was higher in fish fed the COD30 diet than in those fed the COD and COD60 diets, while total alkaline proteases were lower in fish fed the COD30 diet than in those fed the control and COD diets. This pattern may reflect differential regulation of individual proteases, and is in line with meta-analytic evidence showing that the relationship between trypsin and total alkaline protease activity is species-dependent and not necessarily positive (Solovyev et al. 2023). Given the higher protein digestibility observed in COD30, this enzymatic profile suggests a more targeted and efficient proteolytic response, rather than a reduction in digestive capacity, potentially driven by changes in protein accessibility. Comparable induced diet modulation of specific protease activities has been reported in European sea bass (García-Meilán et al. 2023).

Previously, in European seabass, it was also shown that dietary inclusion of non-treated *Gracilaria* spp., *Ulva* spp., or *Fucus* spp. at 2.5% and 7.5% did not affect α-amylase, trypsin, and chymotrypsin activity, while lipase activity decreased only in the *Ulva* spp. diets (Peixoto et al. 2016). Conversely, dietary inclusion of 5% enzymatically hydrolyzed *Rugulopteryx okamurae* increased trypsin and alkaline protease activity (Vizcaíno et al. 2024).

In the present study, the dietary inclusion of 7.5% untreated *C. tomentosum* did not compromise the growth performance of European seabass juveniles. Furthermore, dietary inclusion of C. tomentosum pre-treated with alkaline conditions for 30 min improved performance compared to the control diet. Extending the pre-treatment for 60 min, however, did not yield any additional benefits, resulting in growth performance comparable to that of the control. Previous studies have shown that the effects of macroalgae

inclusion on fish growth performance depend on the pre-treatment method. Enhanced growth performance of European seabass has been reported in diets containing 5% *Ulva rigida* subjected to solid-state fermentation with *Aspergillus ibericus*, whereas in the same study, a low solid-loading alkaline treatment (1:10, w/v) of *U. rigida* did not improve growth performance (Fernandes et al. 2022). In that case, the alkaline hydrolysis of *U. rigida* produced two distinct phases, a liquid and a solid phase, of which only the solid phase was incorporated into the diet. The removal of the liquid phase may have led to the loss of soluble nutrients. By contrast, high solid-loading alkaline hydrolysis used in the present study yielded a single-phase, slurry-like hydrolysate that was fully incorporated into the experimental diets. This approach preserved soluble compounds that might otherwise have been lost, potentially explaining the enhanced growth performance observed in fish fed the COD30 diet. Other research has also shown that the type and intensity of macroalgae pre-treatment can markedly influence fish growth responses. For example, the dietary incorporation of 10% of autoclaved (121 °C for 30 min) *Ulva* sp. meal reduced rainbow trout growth performance compared to non-autoclaved meal (Güroy et al. 2013), and the dietary incorporation of 10% solid-state fermentation *Gelidium* sp. by-product with *A. ibericus* did not enhance the European seabass growth performance compared to the untreated *Gelidium* sp. based diet (Ferreira et al. 2024).

Feed intake was not affected by dietary inclusion of *C. tomentosum* compared with the control. However, feed efficiency and protein efficiency ratio were higher in fish fed the COD30 diet than in those fed the control diet. Moreover, COD30 resulted in higher feed intake and PER than COD60. This agrees with the higher protein digestibility observed for the COD30 diet, suggesting that improved nutrient digestibility contributed to the improved performance of fish fed this diet. The high-solid-loading hydrolysis, which retained the soluble fraction, may also have contributed to the higher feed utilization efficiency observed in the diet COD30. In contrast, extending the pre-treatment to 60 min (COD60 diet) reduced feed intake and feed utilization efficiency, likely due to excessive thermal processing that affected both nutrient availability and feed palatability. Excessive heating may have altered the flavor of the ingredients, thereby reducing voluntary feed intake, as previously observed for other plant-based ingredients (Peres et al. 2003; Glencross et al. 2007b). In contrast, dietary incorporation of *U. rigida* pre-treated by alkaline hydrolysis under a low solid loading (1:10, w/v) reduced feed and protein utilization in European seabass (Fernandes et al. 2022).

The whole-body composition of the fish was unaffected by the experimental diets. Previous studies have also reported that the dietary inclusion of untreated or treated macroalgae at low inclusion levels has little effect on the whole-body composition of European sea bass (Valente et al. 2006; Fernandes et al. 2022). Similar trends have been reported in other fish species, including common carp (*Cyprinus carpio*) fed *U. rigida-based* diets (Diler et al. 2007) or star snapper (*Lutjanus stellatus*) fed a diet containing *Ulva lactuca* (Zhu et al. 2016), where no significant changes in whole-body composition were observed. However, in Nile tilapia, the dietary inclusion of 10–20% *Ulva* spp. reduced whole-body protein and lipid content (Marinho et al. 2013), suggesting that both species and inclusion level may influence the impact of macroalgae inclusion on whole-body composition.

In the present study, the hepatosomatic index of fish fed the COD and COD60 diets was lower than that of the control group. Previous studies have suggested that dietary inclusion of macroalgae may improve liver function by reducing hepatic fat accumulation (Güroy et al. 2013; Natify et al. 2015; Hussein 2017; Elsharkawy et al. 2022). However, contradictory findings have also been reported. No effects on hepatosomatic index were found in seabream fed 5% *Chondrus crispus* (Guerreiro et al. 2019), while an increase was observed in Nile tilapia fed 30% *Hypnea musciformis* (Nur et al. 2020).

The viscerosomatic index was also reduced in fish fed the COD60 diet. This reduction suggests decreased energy storage, which aligns with the reduced energy retention observed in this diet. Similar decreases in the viscerasomatic index have been observed in rainbow trout fed *U. lactuca*-based diets (Güroy et al. 2013) and Nile tilapia fed *Sargassum* sp.-based diets (Natify et al. 2015).

The plasma metabolites measured in this study were within the reference range for European seabass (Peres et al. 2014) and were not affected by the diets. Although diets including pre-treated *C. tomentosum* showed higher fiber digestibility, plasma glucose levels measured 4 h post-prandially were similar among groups. This may be related to the carbohydrate composition of *Codium* spp., which consists of complex sulfated polysaccharides (Fernández et al. 2014), with limited effects on postprandial glucose. Similar results have been reported for Nile tilapia and largemouth bass (*Micropterus salmoides*) (Abd-elaziz and Khakafakka 2015; Kong et al. 2024), while reduced plasma glucose levels have been observed for seabream fed *Ulva* spp. based diets (Vizcaíno et al. 2016; Guerreiro et al. 2019). Plasma lipid levels also remained unchanged, consistent with similar lipid digestibility and lipase activity observed. In other studies, macroalgae have been reported to either reduce (Kong et al. 2024) or increase (Wang et al. 2019) plasma triglycerides and cholesterol levels, reflecting differences in macroalgae composition and the presence of bioactive polysaccharides, such as fucoidans with potencial lipid-lowering properties (Kong et al. 2024).

Conclusion

This study demonstrated that pre-treating *C. tomentosum* with high-solid loading alkaline autoclave hydrolysis for 30 min did not impair feed intake, but rather improved protein and amino acid digestibility, growth performance, and feed and protein utilization efficiency in European seabass. However, extending the pre-treatment to 60 min negatively affected nutrient digestibility and feed intake, resulting in reduced growth performance and feed utilization efficiency. These results demonstrate that high-solid-loading alkaline hydrolysis is a practical and sustainable method for enhancing the nutritional value of *C. tomentosum* while reducing water and energy use during processing. Nevertheless, as only one inclusion level (7.5% on a dry matter basis) was tested, future studies should evaluate higher inclusion levels to assess the scalability and limits of these benefits, as well as their effects on fish well-being and their applicability to other macroalgae.

Author contribution Helena Peres, Aires Oliva-Teles, and Isabel Belo contributed to the general conception and design of the study. The parameters used in this study were conceptualized by Helena Peres and Aires Oliva-Teles. Material preparation, experimental studies, data collection, and analysis were performed by Catarina Ramos-Oliveira, Rui Magalhães, Lúcia Vieira, and Helena Fernandes. The first draft of the manuscript was written by Catarina Ramos-Oliveira. Helena Peres, Aires Oliva-Teles, and Isabel Belo commented on the previous versions. All authors have read and approved the final manuscript. Supervision by Helena Peres, Aires Oliva-Teles, and Isabel Belo, and funding from Helena Peres.

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Data availability Data can be made available by the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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