Aquaculture Reports 34 (2024) 101877

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Effects of feeding European seabass (*Dicentrarchus labrax*) juveniles with crude, hydrolysed and fermented biomass of the invasive macroalga *Rugulopteryx okamurae* (Ochrophyta)

A.J. Vizcaíno [a,*], M.I. Sáez [a], A. Galafat [a], R. Galindo-Melero [b], E. Perera [c], I. Casal-Porras [b], E. Zubía [d], J. Vega [c,e], F.L. Figueroa [e], T.F. Martínez [a], J.A. Martos-Sitcha [b], F.J. Alarcón [a,f]

[a] *Departamento de Biología y Geología, Ceimar-Universidad de Almería, 04120 Almería, Spain*
[b] *Departmento de Biología, Facultad de Ciencias del Mar y Ambientales, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI·MAR), Universidad de Cádiz, Puerto Real, 11510 Cádiz, Spain*
[c] *Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC), Campus Universitario Rio San Pedro s/n, Puerto Real, 11519, Spain*
[d] *Departamento de Química Orgánica, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Puerto Real, 11510 Cádiz, Spain*
[e] *Universidad de Málaga, Instituto Andaluz de Biotecnología y Desarrollo Azul (IBYDA), Centro Experimental Grice Hutchinson, Lomas de San Julián, 2, 29004 Málaga, Spain*
[f] *LifeBioencapsulation S.L., El Alquián, 04131 Almería, Spain*

A R T I C L E I N F O

Keywords:
Rugulopteryx okamurae
Aquafeeds
Enzymatic hydrolysis
Fermentation
Muscle composition
Digestive functionality
Dicentrarchus labrax

A B S T R A C T

The increasing expansion of the invasive Asian alga *Rugulopteryx okamurae* along the Andalusian coasts is a major challenge to marine biodiversity, and urgent coordinated measures are required for its removal or elimination. Among the different actions, the biotechnological valorisation could be a strategy for the management of alien biomass within the frame of the Blue Economy. This work evaluates the potential of *R. okamurae* biomass, either raw or processed by biotechnological treatments based on enzymatic hydrolysis and fermentation processes, to be used as a dietary ingredient in juvenile seabass (*Dicentrarchus labrax*). These treatments, intended primarily to improve the nutrient availability, might also decrease the content of undesirable and potentially harmful algal metabolites, such as the diterpenoid dilkamural. To this end, a 90-day feeding trial was carried out using four experimental diets containing 5% (w/w) crude or processed *R. okamurae* biomass. The results obtained revealed that the inclusion in the diet of raw *R. okamurae* caused adverse effects on fish growth, yielding lower values than those of the control group ($p < 0.05$), although this detrimental effect was negligible when the algae was processed prior to its inclusion in the experimental feeds. In terms of muscle composition, diets supplemented with the algal biomass induced an increase in protein content, especially in fish fed with pre-treated biomass. Qualitative differences ($p < 0.05$) were also found in muscle fatty acid profile, with a significant increase in ARA and DHA contents in fish fed on algae-supplemented diets. A significant reduction ($p < 0.05$)in muscle and liver lipid oxidation was evidenced in fish fed the algae-enriched diets, especially with treated biomass. Finally, 5% *R. okamurae* raw biomass caused negative effects on gut functionality, although these effects were not observed when the algal biomass was previously processed. In conclusion, the results revealed the potential of *R. okamurae* as a functional ingredient for the feeding of juvenile sea bass, provided that the biomass is pre-treated, and corroborated the efficacy of a low-cost biotechnological treatment as a valorisation method for the biomass of this invasive algae

1. Introduction

The increasing expansion of the invasive Asian alga *Rugulopteryx okamurae* on the Andalusian coasts is recognized as a major threat to marine biodiversity, profoundly affecting the structure and functioning of native botanical and faunal communities (Steen et al., 2017), as well as the accumulation of thousands of tons of wrack deposits on the beaches (García-Gómez et al., 2020).

* Corresponding author.
E-mail address: avt552@ual.es (A.J. Vizcaíno).

https://doi.org/10.1016/j.aqrep.2023.101877
Received 19 September 2023; Received in revised form 29 October 2023; Accepted 3 December 2023

Therefore, urgent coordinated measures are required for its removal or elimination by searching sustainable uses of the large amount of biomass available. In this sense, efforts aimed at the valorisation of this biomass, by means of biotechnological procedures, is a burgeoning research field. Among the potential applications of the algal biomass, its use as feed ingredient in animal nutrition, and especially in aquaculture, could be of interest, owing to the promising results of macroalgae as ingredient in aquafeeds (Wan et al., 2019). Indeed, there is plenty of scientific literature related to the use of algae-supplemented diets for fish and their potential beneficial effects on different aspects of fish growth and development, muscle composition, and digestive functionality (Vizcaíno et al., 2016, 2019; Peixoto et al., 2019; Sáez et al., 2020).

From a nutritional point of view, macroalgae are recognised as a sustainable source of protein and lipids, but in recent years the interest has been directed towards their use as a source of bioactive compounds, such as polysaccharides, pigments, antioxidants, polyphenols, and vitamins (Vizcaíno et al., 2020). Specifically, brown algae of the family Dictyotaceae, which includes *Rugulopteryx* species, have been shown to contain, in addition to fucoxanthin as a typical carotenoid of brown algae, an array of secondary metabolites of the terpenoid class, which displays antimicrobial, anti-inflammatory, antifungal or anti-cancer properties (De Paula et al., 2011; Chen et al., 2018). However, and similar to what has been observed for other families of macroalgae, there are some technical gaps as the low digestibility of the cell wall (Wan et al., 2019; Domozych et al., 2012; Demarco et al., 2022) or the presence of anti-nutritional factors (Vizcaíno et al., 2020) that could limit their use in aquafeeds, even at low inclusion levels. Moreover, *R. okamurae* from the coasts of the south of Spain has been shown to contain high amounts of dilkamural, a secondary metabolite toxic for some herbivores (Casal-Porras et al., 2021). In view of these limitations, it is reasonable to consider the need for strategies aimed at improving the bioavailability of the nutrients, as well as reducing the adverse effects of anti-nutritional substances in this algal biomass, as a necessary step prior to its possible use in aquafeeds. One of the available strategies is enzymatic lysis, which has been widely studied on algal biomass due to its high biological specificity, adequate operating conditions, and low energy and investment costs (Günerken et al., 2015). Previous studies pointed out the effectiveness of these treatments as promising strategies for algal cell wall disruption, improving the bioavailability of nutrients by breaking the glycosidic bonds between the monomers of structural polysaccharides (Galafat et al., 2020). Applied to macroalgae, enzymatic hydrolysis could enhance nutrient bioavailability by breaking the glycosidic linkages between the carbohydrates of the indigestible polysaccharide algal cell wall, as well as remove antinutritive factors that limit their use at high inclusion level (Vizcaíno et al., 2020). In fact, the processing land-based crops, such as soybean or lupin, through the use of exogenous enzymes has overcome the effects of antinutritional factors and digestibility issues, this enabling higher inclusion levels of these ingredients in diets for less tolerant fish species (Wan et al., 2019).

Alternatively, microbial fermentation is another simple and economical method that might increase the nutrient content and the digestibility of algal biomass. This procedure promotes the hydrolysis of polymers from raw materials, the synthesis of bioactive molecules, as well as the degradation of toxic or antinutritional factors. Indeed, previous research has shown that the use of microorganisms such as *Lactobacillus* spp. and *Saccharomyces cerevisiae* improves the nutritional quality and digestibility of macroalgae (Felix and Brindo, 2014; Aslamyah et al., 2017).

In this perspective, the present work hypothesises that an adequate biotechnological treatment of algal biomasses, including not only enzymatic hydrolysis and fermentation processes, but also a sequential treatment of enzymatic hydrolysis followed by fermentation, may represent a valuable strategy to increase the nutrient bioavailability and functional properties compared to the untreated raw material. In addition, this procedure could be a suitable tool for the circular bioeconomy, as it is possible to convert solid waste, such as the tonnes of of *R. okamurae* wrack deposits on beaches, into value-added products for aquaculture. To this end, raw and processed biomass of *R. okamurae* were evaluated as a potential dietary ingredient in practical diets for Mediterranean fish, using European seabass (*Dicentrarchus labrax*) as animal model, through the assessment of the effects on fish growth, muscle composition, oxidative status, skin pigmentation, intermediary metabolism and digestive functionality.

2. Material and methods

2.1. Algal biomass

Biomass (30 kg fresh weight) of *Rugulopteryx okamurae* ((E.Y.Dawson) I.K. Hwang,W.J. Lee & H.S. Kim) Dictyotales, Ochrophyta) was collected at 3 m depth from La Caleta de Tarifa (Cádiz, Spain). The biomass was washed in fresh water to remove salt and sand particles, and then was dried in a forced ventilation chamber (Airfrío, Almería, Spain) at 30 ºC for 12 h. Once dried, it was ground in a laboratory pulveriser (100 UPZ, Hosokawa Alpine, Germany) and sieved through a 0.2 mm mesh to obtain homogeneous powder.

In addition to being used as raw material, part of the algal biomass was subjected to three biological treatments (enzymatic hydrolysis, microbial fermentation and a sequential combination of both) before being incorporated into the experimental diets, with the aim of assessing the possible effects of such treatments on the nutritional value of the algae. The pre-treatments were carried out by LifeBioencapsulation S.L. (Almería, Spain). Briefly, for the enzymatic hydrolysis, *R. okamurae* dry biomass was suspended (100 g dry weight L^{-1}) in 50 mM sodium citrate buffer (pH 5.0) and incubated at 50 ºC under continuous agitation for 24 h in presence of a blend of enzymes (xylanase 20,000 U g^{-1}; glucanase 30,000 U g^{-1}; cellulase 10,000 U g^{-1}, and protease 10,000 U g^{-1}) providing a 0.05 enzyme to algae ([E]/[S]) ratio. For microbial fermentation, a wet sludge of *R. okamurae* containing 100 g L^{-1} (DW) were transferred to a fermentation vessel and inoculated with 10^8 CFU g^{-1} *Saccharomyces cerevisiae* and 10^8 CFU g^{-1} *Bacillus subtilis* provided by the Spanish Type Culture Collection. Dextrose (5% w/w of the base material) was used as sugar substrate. The fermentation was carried out at 40 ºC until pH reached 4.5. Values of pH in the range between 4.0 and 5.0 are desired in order to prevent possible microbial spoilage, while not interfering with the voluntary intake of the feed elaborated afterwards. In addition, a third batch of pre-treated seaweed was obtained by a sequential combination of both processes, carrying out the enzymatic hydrolysis followed by fermentation according to the described protocols.

For the analysis of the diterpene dilkamural (DK) in the different types of algal biomasses (crude, hydrolysed, fermented, hydrolysed + fermented), triplicate samples (0.5 g) were withdrawn from each algal biomass, treated with 3 mL acetone/methanol (1:1, v/v) and subjected to sonication for 5 min. After centrifugation (5 min, 10000 g) the supernatants were collected, and the pellets subjected to another cycle of extraction. A total of 4 cycles of extraction were performed on each sample. The supernatants were combined and the solvent evaporated at reduced pressure in a rotary evaporator. The resulting extract was suspended in 1 mL methanol/water (9:1, v/v) and transferred onto a solid phase extraction (SPE) cartridge (SUPELCO DSC18, 500 mg/3 mL) preconditioned with 1 mL methanol/water (9:1, v/v). The cartridge was eluted with 8 mL of the same solvent, and the resulting solution was evaporated to dryness under reduced pressure in a rotary evaporator. A sample of this final extract (15–20 mg) was dissolved in 0.6 mL deuterated methanol (CD_3OD) and analysed by ^{1}H nuclear magnetic resonance (^{1}H NMR) (Fig. S1). The spectra were recorded on a 500 MHz Bruker spectrometer. Chemical shifts were referenced using the solvent signal at δ 3.30. For quantitative ^{1}H NMR analysis, 1,3,5-trimethoxybenzene (Sigma-Aldrich Trace CERT) was added as internal standard and the spectra were recorded using a pulse width of 8 μs (90º), relaxation delay of 30 s and acquisition time of 3 s. The signals used for

quantification were those at δ 9.60 (1 H), assigned to the aldehyde proton of 2-deacetoxy-2,3-didehydrodilkamural (DKE) (Casal-Porras et al., 2021), and at δ 6.07 (3 H) corresponding to the aromatic protons of the internal standard. The start and end points for integration of these peaks were selected manually.

2.2. Experimental diets

Five iso-nitrogenous (42.0% crude protein, DW) and isolipidic (18.0% crude lipid, DW) experimental diets were formulated and elaborated by the Universidad de Almeria facilities (Experimental feeds Service; http://www.ual.es/stecnicos_spe) using standard aquafeed processing procedures to obtain 2 and 3 mm pellets. The five experimental diets were formulated to mimic the basic composition of commercial feeds for the European seabass. An algae-free diet was used as control (CT) and four additional diets were designed including 5% of each one of the *R. okamurae* biomass named as CRU (5% of the crude macroalgae, without any biotechnological treatment); ENZ (5% of enzymatic hydrolysed biomass); FER (5% of fermented biomass) and Section (5% of sequential hydrolysed and fermented biomass). Briefly, all the ingredients were finely ground and mixed in a vertical helix ribbon mixer (Sammic BM-10, 10-L capacity, Sammic, Azpeitia, Spain) and then, the fish oil and diluted choline chloride were added to the blend. All the ingredients were mixed for 15 min, and water (350 mL per 1 kg of mass ingredient) was added to obtain a homogeneous dough. The dough was extruded using a two-screw extruder (Evolum 25, Clextral, Firminy, France). The extruder barrel consisted of four chambers that were maintained with a temperature profile (from inlet to the extruder die head) of 95 ∘C, 98 ∘C, 100 ∘C, and 110 ∘C, respectively. The formulation and the fatty acid profile of the experimental diets are shown in Tables 1 and 2.

2.3. Feeding trial and sampling protocol

The feeding trial was carried out at CTAQUA experimental facilities (El Puerto de Santa María, Cádiz, Spain. Spanish Operational Code REGA ES110270000411). Three hundred seabass (*D. labrax*) juveniles (24–25 g, initial body weight) were obtained from commercial sources (CUPIBAR, Chiclana de la Frontera, Cádiz). After a 7-day acclimation period, fish were randomly distributed in fifteen 100-L tanks (n = 20 fish per tank, 60 fish per experimental diet) and were fed ad libitum with the experimental feeds 3 times a day and 6 days per week. Each feed was tested in triplicate (5 diets × 3 tanks each) for 13 weeks. The trial was conducted from April to July in a recirculation aquaculture system (RAS), under constant temperature (21 ± 0.5 °C) and an oxygen content of outlet water higher than 85% saturation. Day-length followed the natural changes at our latitude (36º35'06''N; 06º13'48''W; Cádiz, Spain).

Fish were kept and handled following the guidelines for experimental procedures in animal research of the Ethics and Animal Welfare Committee of the University of Cadiz, according to the Spanish (RD53/2013) and European Union (2010/63/UE) legislation. The Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 04/04/2019/056).

At the end of the trial (day 91), overnight fasted fish were deeply anaesthetised with an overdose of 2-phenoxyethanol (1 mL L^{-1} and individually weighed. Eight fish per tank (24 per experimental treatment) were randomly selected, skin colour was immediately recorded and then fish were sampled for blood collection. Blood was drawn from caudal vessels with heparinised syringes, centrifuged at 3,000g for 20 min at 4 ºC, and plasma samples were snap-frozen in liquid nitrogen and stored at − 80 ºC until biochemical analysis. Prior to tissue collection, fish were killed by cervical section. Sampled fish were dissected, and muscle, liver and digestive tract were removed. Muscle samples (24 animals per treatment) were taken for thiobarbituric acid-reactive substances (TBARS) determinations, and the rest were freeze-dried and stored at − 20 °C for further analysis of muscle proximate composition. Then, muscle samples were randomly pulled in six different batch per treatment. Representative samples of liver were rapidly taken, snap-frozen in liquid nitrogen, and stored at − 80 ºC until biochemical analyses. For the enzymatic analysis, intestines from five fish were randomly pooled to obtain two different extracts from each experimental tank (6 different extracts per treatment). Briefly, intestines were manually homogenised in distilled water to a final concentration of 0.5 g mL^{-1}. The homogenate was centrifugated (10,000g, 12 min, 4 °C) and the supernatant was stored at − 20 °C for further enzymatic analysis. Finally, intestine and liver samples of three specimens from each tank were collected for examination by light microscopy (LM), and transmission (TEM) and scanning (SEM) electron microscopy.

Table 1
Ingredient composition of the experimental diets.

	CT	CRU	ENZ	FER	SEC
Ingredient composition (g kg^{-1} on dry matter basis)					
Fishmeal LT94[1]	120.0	120.0	120.0	120.0	120.0
Krill meal[2]	30.0	30.0	30.0	30.0	30.0
Crude algae[3]		50.0			
Hydrolysed algae[3]			50.0		
Fermented algae[3]				50.0	
Hydrolysed-fermented algae[3]					50.0
Wheat gluten[4]	133.0	133.0	133.0	133.0	133.0
Soybean meal[5]	250.0	250.0	250.0	250.0	250.0
Soybean protein concentrate[6]	100.0	100.0	100.0	100.0	100.0
Fish oil[7]	92.0	92.0	92.0	92.0	92.0
Soybean oil[8]	43.0	42.0	42.0	42.0	42.0
Wheat meal[9]	165.0	116.0	116.0	116.0	116.0
Lysine[10]	15.0	15.0	15.0	15.0	15.0
Methionine[11]	6.0	6.0	6.0	6.0	6.0
Betain[12]	5.0	5.0	5.0	5.0	5.0
Vitamin and mineral premix[13]	20.0	20.0	20.0	20.0	20.0
Vitamin C[14]	1.0	1.0	1.0	1.0	1.0
Guar gum[15]	20.0	20.0	20.0	20.0	20.0
Proximate composition (g kg^{-1} on dry matter basis)					
Crude protein	433.0	430.0	424.0	421.0	425.0
Crude lipid	162.0	160.0	165.0	168.0	165.0
Ash	68.0	74.0	76.0	75.0	76.0
Gross energy (MJ/kg)[16]	221.0	220.0	220.0	221.0	220.0

Dietary codes: CT: algae-free control diet; CRU: 5% inclusion of crude R. okamurae biomass. ENZ: 5% inclusion of enzymatically-hydrolysed R. okamurae biomass. FER: 5% inclusion of fermented R. okamurae biomass. SEC: 5% inclusion of sequential hydrolysis and fermented R. okamurae biomass. 169.4% crude protein, 12.3% crude lipid (Norsildemel, Bergen, Norway). 2purchased from Bacarel (UK). CPSP90 is enzymatically pre-digested fishmeal. [3]Fonseca et al. (2023); 478% crude protein (Lorca Nutrición Animal SA, Murcia, Spain). 548% crude protein, 8% crude lipid (Lorca Nutrición Animal SA, Murcia, Spain). 6Soybean meal, 65% crude protein, 4% crude lipid (DSM, France). 7AF117DHA (Afamsa, Spain). 8P700IP (Lecico, DE). 9Local provider (Almería, Spain). 10, 11, 12Lorca Nutrición Animal SA (Murcia, Spain). 13LifeBioencapsulation S.L. (Almería, Spain). Vitamins (mg kg-1): vitamin A (retinyl acetate), 2000,000 UI; vitamin D3 (DL-cholecalciferol), 200,000 UI; vitamin E (Lutavit E50), 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2500 mg; vitamin B1(thiamine hydrochloride), 3000 mg; vitamin B2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine (Betafin S1), 50,000 mg. Minerals (mg kg-1): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 18.6%; (186,000 mg); KCl, 2.41%; (24,100 mg); NaCl, 4.0% (40,000 mg). 14TECNOVIT, Spain; 15EPSA, Spain. 16Gross energy was estimated by energetic coefficients (kJ/g) according to Miglavs and Jobling (1989): crude protein, 23.6; crude lipid, 38.9; Nfe, 16.7.

2.4. Muscle proximate composition and fatty acids analysis

Proximate analysis of feed and muscle samples were determined

Table 2
Fatty acid profile (% total fatty acids) of the experimental diets.

	CT	CRU	ENZ	FER	SEC
14:0	2.14	2.19	2.19	2.17	2.19
16:0	17.29	17.35	17.43	17.26	17.39
16:1n7	3.19	3.21	3.21	3.18	3.22
16:2n4	0.70	0.70	0.73	0.72	0.72
16:3n4	0.47	0.47	0.46	0.46	0.45
18:0	5.15	5.22	5.22	5.20	5.23
18:1n7	1.94	1.94	1.91	1.91	1.91
18:1n9	18.42	18.27	18.18	18.06	18.16
18:2n6	23.98	23.72	23.63	23.42	23.42
18:3n3	1.19	1.21	1.13	1.09	1.10
18:4n3	0.61	0.71	0.66	0.63	0.69
20:4n6	1.08	1.13	1.15	1.11	1.13
20:4n3	0.40	0.41	0.37	0.35	0.37
20:5n3	4.72	4.74	4.73	4.69	4.73
20:1n9	1.29	1.85	1.59	1.84	1.58
22:5n3	1.15	1.11	1.22	1.18	1.21
22:6n3	12.54	12.62	12.60	12.54	12.65
SFA	24.58	24.76	24.83	24.63	24.81
MUFA	24.84	25.28	24.89	24.99	24.87
PUFA	45.67	45.66	45.49	45.02	45.31
Other	3.74	3.14	3.60	4.18	3.84

according to AOAC (2002) procedures for dry matter and ash. Crude protein (N × 6.25) was determining by elemental analysis (C:H:N) using a Fisons EA 1108 analyser (Fisons Instruments, Beverly, MA, USA). Total lipid content was quantified following the procedure described by Folch et al. (1957) using chloroform/methanol (2:1 v/v) as solvent. Fatty acid profiles of feeds and muscle samples were determined by gas chromatography following the method described in Rodríguez-Ruiz et al. (1998).

2.5. Skin pigmentation

Instrumental colour was determined on the right side of the dorsal fish skin by L*, a*, and b* system (CIE, 1978), using a Minolta chroma meter CR-400 (Minolta, Japan). The lightness (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 recorded (CIE, 1978).

2.6. Muscle and liver lipid oxidation

Lipid oxidation in muscle and liver samples was estimated by thiobarbituric acid-reactive substances (TBARS) according to the method of Buege and Aust (1978). Briefly, samples (1 g) were homogenized in 4 mL 50 mM NaH_2PO_4, 0.1% (v/v) Triton X-100 solution and centrifuged (10, 000g, 20 min, 4 °C). Supernatants were mixed (1:5 v/v) with 2-thiobarbituric acid (TBA) reagent (0.375% w/v TBA, 15% w/v TCA, 0.01% w/v 2,6-di-tert-butyl-4-methylphenol (BHT) and 0.25 N HCl) and heated for 15 min. Finally, the mixture was centrifuged (3600g, 10 min, 4 °C), and the absorbance of supernatants was measured at 535 nm. The amount of TBARS was expressed as mg of malondialdehyde (MDA) per kg of tissue after comparing with a MDA standard (Sáez et al., 2020).

2.7. Biochemical parameters on plasma and liver

Plasma cortisol levels were measured with a commercial cortisol enzyme immunoassay kit (DetectX®, K003, Arbor Assays). Glucose, lactate, triglycerides and cholesterol levels in plasma were measured using commercial kits (Spinreact, St. Esteve de Bas, Girona, Spain) adapted to 96-well microplates. Plasma total protein concentration was determined with a BCA protein assay kit (PierceTM, Thermo Fisher Scientific, USA, #23225) using bovine serum albumin (BSA) as standard. Prior to metabolites assessment in liver, frozen tissues were homogenized by ultrasonic disruption in 7.5 volumes ice-cold 0.6 N perchloric acid, neutralised with 1 M KCO_3, centrifuged (30 min, 3220g, 4 ºC), and then supernatants isolated to determine tissue metabolites. Tissue triglycerides levels were determined spectrophotometrically with commercial kits (Spinreact, see above). Tissue glycogen concentration was quantified using the method by Keppler and Decker (1974). Glucose obtained after glycogen hydrolysis with amyloglucosidase (Sigma-Aldrich A7420) was determined with a commercial kit (Spinreact) as described before. All assays were performed using a PowerWave™ 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using the KCjunior™ data analysis software for Microsoft®.

2.8. Activity of metabolic enzymes in liver

Frozen tissues for enzyme activity assays were homogenized by ultrasonic disruption in 10 volumes of ice-cold homogenisation buffer (50 mM imidazole, 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM sucrose; pH 7.5). Homogenates were centrifuged (30 min, 3220g, 4 ºC), and supernatants stored at − 80 ºC for further analysis. The assays of several enzymes involved in glycogenolysis (GPase: glycogen phosphorylase, EC 2.4.1.1), glycolysis (HK: hexokinase, EC 2.7.1.1; PK: pyruvate kinase, EC 2.7.1.40), gluconeogenesis (LDH: lactate dehydrogenase, EC 1.1.1.27; FBP: fructose 1,6-bisphosphatase, EC 3.1.3.11), and lipid metabolism (HOAD: 3-hydroxyacyl-CoA dehydrogenase, EC 1.1.1.35) were performed as previously described in Perera et al. (2020). Enzyme activities were determined using a PowerWaveTM 340 microplate spectrophotometer and KCjuniorTM data analysis software for Microsoft®. Activities were expressed as specific activities per mg of protein in the homogenates (U·mg $prot^{-1}$). Total protein in liver samples was assayed in duplicate, as described above for plasma samples.

2.9. Digestive enzyme activity

Total alkaline protease activity was determined according to Alarcón et al. (1998) using buffered 5 g L^{-1} casein (50 mM Tris–HCl, pH 9.0) as substrate, defining one unit of activity (UA) as the amount of enzyme that released 1 μg of tyrosine per min (extinction coefficient for tyrosine of 0.008 μg^{-1} mL^{-1} cm^{-1}). Trypsin and chymotrypsin activities were quantified using as substrate 0.5 mM BAPNA (N-α-benzoyl-DL-arginine-4-nitroanilide) (Erlanger et al., 1961) and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-P-nitroanilide) (DelMar et al., 1979), respectively. Leucine aminopeptidase was assayed using buffered 2 mM L-leucine-p-nitroanilide (LpNa) (100 mM Tris–HCl buffer, pH 8.8) according to Pfleiderer (1970). Alkaline phosphatase was determined using buffered p-nitrophenyl phosphate (pH 9.5) (Bergmeyer, 1974). One UA of trypsin, chymotrypsin and leucine aminopeptidase was stablished as the amount of enzyme that releases 1 μmol p-nitroanilide per min (extinction coefficient 8800 M cm^{-1} at 405 nm), while for alkaline phosphatase, one UA was defined as the amount of enzyme releasing 1 μg tyrosine min^{-1} (extinction coefficient of p-nitrophenol, 17, 800 M cm^{-1} at 405 nm). All assays were performed in triplicate, and specific enzymatic activity was expressed as UA g $tissue^{-1}$.

In addition, a substrate-SDS-PAGE electrophoresis gels of the intestinal extracts was carried out following the method describe for Laemmli (1970) using 12% polyacrylamide gels (100 V per gel, 45 min, 4 °C). Zymograms revealing protease active bands were carried out following the method described by Alarcón et al. (1998).

2.10. Histological analysis of intestine and liver

Intestine and liver samples were fixed with phosphate-buffered formalin (4% v/v, pH 7.2) for 24 h. Fixed samples were dehydrated and embedded in paraffin according to standard histological protocols. After that, transversal Section (5 μm) of the intestine were obtained which were stained with hematoxylin-eosin (H&E). The stained

preparations were examined under light microscope (OLYMPUS IX51) equipped with a digital camera CC 12 (Olympus Soft Imaging Solutions GmbH, Muenster, Germany). The images were analysed using UTHSCA ImageTool software for measuring fold length, total enterocyte height, *lamina propria* thickness and the width of submucosa, muscular and serosa layers (50 independent measurements per treatment).

2.11. Electron microscopy study

Intestinal sections were processed by electron microscopy analysis following the methodology described in Vizcaíno et al. (2014). Briefly, for transmission electron microscopy (TEM) (Carl Zeiss Libra 120 Plus (EELS), Germany), samples were fixed for 4 h at 4 °C using buffered 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde (phosphate buffer saline (PBS), pH 7.5). After washing with PBS, a post-fixation with osmium tetroxide (20 g L^{-1}) was carried out prior to tissue dehydration by consecutive immersions in gradient ethanol solutions (from 50% to 100%, v/v). Dehydrated samples were embedded for 2 h in 100% (v/v) ethanol and Epon resin (1:1) under continuous shaking and then included in pure Epon resine for 24 h, and polymerized at 60 °C. Finally, the ultrafine cuts were placed on a 700 Å cooper mesh and stained with uranile acetate and lead citrate. The observation of the samples was performed with a transmission electron microscope (Zeiss 10 C, Carl Zeiss, Barcelona, Spain) at 100 Kv and visualization fields were recorded (× 16,000 magnification). For SEM analysis, samples were fixed in phosphate-buffered formalin (4% v/v, pH 7.2) for 24 h, after washing with 1% S-carboxymethyl-L-cysteine (Sigma) to remove epithelial mucus. Afterwards, samples were dehydrated with graded ethanol, followed by critical point drying (CDP 030 Critical point dryer, Leica Microsystems, Madrid, Spain) using absolute ethanol and CO_2 as the intermediate and transition fluids, respectively. Dried samples were mounted on supports, fixed with graphite (PELCO® Colloidal Graphite, Ted Pella Inc., Ca, USA), and then gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). Finally, all samples were observed with a scanning electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). TEM and SEM micrographs were analysed using UTHSCA ImageTool software for morphometric analysis to determine the microvilli length (mL), the microvilli diameter (MD), the number of microvilli over 1 μm distance, the enterocyte apical area (EA), and the total absorption surface per enterocyte (TAS) (50 independent measurements per treatment), as described in Vizcaíno et al. (2014).

2.12. Statistical analyses

Results are expressed as mean ± standard deviation (SD). In order to test data normality and variance homogeneity, the Kolmogorov-Smirnov's test and Levene's F-test were used, respectively. Data with parametric distribution were analysed using a one-way analysis of variance (ANOVA) and the significant differences between treatments ($p < 0.05$) were determined using Tukey's multiple comparison test. Data with nonparametric distribution were analyzed using Kruskal–Wallis test, and significant differences were determined using Box and Whisker Plot graphs. Data in percentage were arcsin ($x^{1/2}$) transformed. All statistical tests were performed using Statgraphics Plus 4.0 (Rockville, Maryland, USA) software.

3. Results

3.1. Dilkamural in algae extracts

The content of 2-deacetoxy-2,3-didehydrodilkamural; DKE (% w/w) was 0.55 ± 0.08^{a} in crude algal biomass, 0.04 ± 0.01^{b} in algal biomass derived from enzymatic hydrolysis, and 0.18 ± 0.04^{c} in algal biomass derived from fermentation treatment, while dilkamural (DK) was not detected in biomass derived from sequential hydrolysis and fermentation processes (Fig. S1) (a-c: different letter superscripts stand for significant differences; df = 2, NS = 0.21, F = 91.43, $p < 0.01$).

3.2. Growth performance

There was no mortality during the feeding trial. All fish groups grew allometrically from 24 to 25 g to 65–77 g depending on the dietary group, although significant differences on body weight were evidenced at the end of the feeding assay. Overall, fish fed on diet including *R. okamurae* biomass showed a significantly lower final body weight compared with CT group, although SEC group reached values similar to those obtained in fish fed the algae-free diet (Fig. 1).

3.3. Muscle proximate composition and fatty acid profile

Muscle proximal composition is shown in Table 3. Protein content increased significantly in fish fed with the diets supplemented with the enzymatically hydrolysed and sequentially-treated *R. okamurae* biomasses (ENZ and SEC, respectively), while no significant changes were observed for total lipids, moisture and ash ($p > 0.05$).

Muscle fatty acid profile of juvenile seabass is shown in Table 4. The results revealed significant differences among control and fish fed on diets containing algal biomass ($p < 0.05$). Polyunsaturated fatty acids (PUFA) were the predominant fraction, reaching concentrations ranging from 40% to 43% total fatty acids, followed by saturated fatty acids (SFA, 24%) and monounsaturated fatty acids (MUFA) with concentrations between 24% and 26%. Considered individually, the predominant fatty acids were palmitic acid (C16:0) within the SFA fraction, with a content around 17% of the total fatty acids; and oleic acid (C18:1n9) among the MUFAs (ranging from 18% to 21%). Regarding the PUFA fraction, a significant increase was observed in fish fed with the diets supplemented with *R. okamurae* ($p < 0.05$). It is worth mentioning the increase observed in arachidonic acid (ARA, C20:4n6) and docosahexaenoic acid (DHA, C22:6n3) contents in animals fed with those feeds containing algal biomass, compared to those of control group.

3.4. Muscle and liver lipid oxidation (TBARS)

Lipid oxidation measurements in muscle and liver indicated significant differences attributable to the dietary inclusion *R. okamurae* biomass (Fig. 2). Both in muscle and liver samples, the inclusion of *R. okamurae* tended to decrease lipid oxidation. In liver samples, this decrease in TBARS concentration was statistically significant in all the dietary treatments with 5% *R. okamurae* biomass, although the lowest

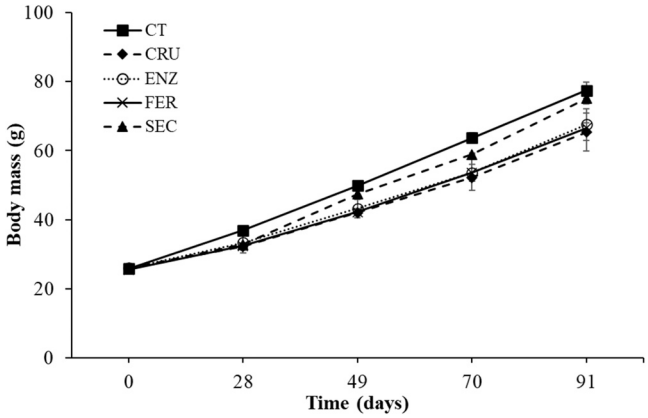

Fig. 1. Time course of changes in body weight of fish fed with the experimental diets (n = 60). Dietary codes: Dietary codes: CT (Control diet); CRU (5% crude biomass of *R. okamurae*); ENZ (5% hydrolysed *R. okamurae* biomass); FER (5% fermented *R. okamurae* biomass); Section (5% sequential hydrolysis and fermented *R. okamurae* biomass).

Table 3
Muscle composition (% dry weight) in *D. labrax* specimens fed on the experimental diets at the end of the feeding trial.

	Total protein	Total lipids	Ash	Moisture
CT	73.41 ± 2.78 a	15.74 ± 0.20	6.41 ± 0.17	76.32 ± 0.57
CRU	74.41 ± 1.28 ab	13.14 ± 0.27	6.66 ± 0.30	76.85 ± 0.41
ENZ	79.31 ± 0.27 b	13.62 ± 0.74	6.43 ± 0.17	75.98 ± 0.61
FER	76.50 ± 0.18 ab	15.27 ± 0.72	7.24 ± 1.52	76.46 ± 0.42
SEC	79.63 ± 0.09 b	13. 77 ± 1.16	6.65 ± 0.40	76.85 ± 0.39
p	*0.0197*	*0.2668*	*0.6385*	*0.2770*

Values are expressed as mean ± SD (n = 6). Dietary codes: Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

values were observed when the algal biomass was pre-treated (ENZ, FER and SEC). In muscle samples, TBARS content was also lower in fish fed algae-supplemented diets, although differences were significant only in FER and SEC groups.

3.5. Skin pigmentation

Regarding instrumental colour determination, no significant differences were observed in any of the colour parameters studied (L*, a* and b*) among the different treatments evaluated (Fig. 3). L* parameter was higher than 50 in all fish. Skin a* parameter yielded negative values, indicating a greenish coloration, whereas b* values were positive, showing a yellowish pigmentation, especially in CRU and FER groups, although differences were not significant.

3.6. Metabolic orchestration in blood and liver

Data on plasma and hepatic parameters are presented in Table 5. Plasma cortisol levels decreased in fish fed with the inclusion of the four types of macroalgal biomasses, although this lowering effect was only significant in those fish fed with FER and SEC diets compared with control group. Dietary supplementation did not alter plasma levels of glucose and protein, whereas a significant increase on plasma lactate was found in both CRU and ENZ lots. Moreover, levels of plasma triglycerides and cholesterol were decreased significantly in fish fed with CRU diet. In liver, no effects of dietary supplementation were found on the storage levels of glucose and glycogen. However, a significant decrease in hepatic triglycerides was detected in fish fed the experimental feeds containing the algal biomass subjected to the sequential pre-treatment (ENZ+FER) compared with those fish from the CRU group.

Regarding hepatic metabolic enzymes (Table 6), no significant differences were observed in the activity of FBP, GPase and PK. On the other hand, a significant increase was observed in the activity of fish fed CRU diet when compared with those fed CTRL (HOAD), ENZ, FER and ENZ+FER (HK), or CTR, FER and ENZ+FER (LDH) groups.

3.7. Digestive enzyme activities

Enzyme activities in the intestinal extracts of seabass juveniles fed the experimental diets are shown in Table 7. In general, the inclusion of *R. okamurae* had a significant effect on the enzyme activity levels evaluated. Fish fed on algae-supplemented diets showed higher levels of total alkaline protease activity than those observed in the CT group, except for CRU group, which showed similar values than those of fish fed on algae-free diet. Trypsin and chymotrypsin activities tended to increase in fish fed *R. okamurae* supplemented diets, although significant differences were observed only in FER and ENZ groups, respectively. Regarding brush border enzymes, a significant decrease ($p < 0.05$) in leucine aminopeptidase and alkaline phosphatase activity levels was observed in fish fed on 5% untreated biomass of *R. okamurae* (CRU) compared to the values observed in CT group. However, this decrease was not observed in fish fed the processed biomasses (ENZ, FER and SEC), showing values similar to those observed in CT group.

Zymograms carried out with intestinal extracts are shown in Fig. 4.

Table 4
Muscle fatty acid composition (% of total fatty acids) in *D. labrax* specimens fed the experimental diets.

	CT	CRU	ENZ	FER	SEC	*p*
14:0	1.99 ± 0.01c	2.05 ± 0.01 b	1.90 ± 0.01 a	2.05 ± 0.01 b	1.85 ± 0.01 a	*< 0.001*
16:0	17.23 ± 0.01 b	17.04 ± 0.04 a	17.41 ± 0.06 bc	17.29 ± 0.04 b	17.53 ± 0.02c	*0.001*
16:1n7	3.55 ± 0.02 bc	3.46 ± 0.01 ab	3.33 ± 0.01 a	3.73 ± 0.09c	3.38 ± 0.04 ab	*0.001*
16:2n4	0.52 ± 0.01 a	0.54 ± 0.01 a	0.53 ± 0.02 a	0.52 ± 0.01 a	0.58 ± 0.02 b	*0.005*
16:3n4	0.46 ± 0.01	0.45 ± 0.01	0.45 ± 0.02	0.45 ± 0.00	0.41 ± 0.01	*0.1405*
18:0	5.24 ± 0.05 b	5.09 ± 0.01 a	5.47 ± 0.01c	5.01 ± 0.01 a	5.45 ± 0.03c	*< 0.001*
18:1n7	2.08 ± 0.001	2.06 ± 0.01	2.06 ± 0.01	2.10 ± 0.01	2.09 ± 0.03	*0.1315*
18:1n9	20.92 ± 0.05	19.73 ± 0.01	18.83 ± 1.6	20.65 ± 0.03	20.36 ± 0.09	*0.1553*
18:2n6	17.52 ± 0.09 ab	18.13 ± 0.26 b	17.84 ± 0.22 ab	17.28 ± 0.10 a	17.58 ± 0.03 ab	*0.0213*
18:3n3	1.13 ± 0.05	1.15 ± 0.02	1.15 ± 0.03	1.12 ± 0.03	1.07 ± 0.21	*0.9275*
18:4n3	0.60 ± 0.02 b	0.60 ± 0.03 b	0.54 ± 0.01 ab	0.54 ± 0.04 ab	0.47 ± 0.01 a	*0.0111*
20:4n6	1.15 ± 0.02 a	1.29 ± 0.02 bc	1.34 ± 0.03c	1.19 ± 0.01 ab	1.38 ± 0.06c	*0.0039*
20:4n3	0.46 ± 0.04	0.43 ± 0.01	0.45 ± 0.05	0.41 ± 0.03	0.34 ± 0.09	*0.2425*
20:5n3	4.15 ± 0.01 ab	4.20 ± 0.05 ab	4.23 ± 0.03 b	4.07 ± 0.06 a	4.11 ± 0.02 ab	*0.0395*
22:5n3	1.15 ± 0.05	1.09 ± 0.01	1.08 ± 0.04	1.08 ± 0.07	1.15 ± 0.34	*0.9664*
22:6n3	14.54 ± 0.07 a	15.22 ± 0.01c	15.94 ± 0.05 d	14.52 ± 0.04 b	15.41 ± 0.05c	*< 0.001*
SFA	24.55 ± 0.05 a	24.18 ± 0.03 a	24.78 ± 0.08 b	24.35 ± 0.03 a	24.83 ± 0.01 b	*< 0.001*
MUFA	26.55 ± 0.06	25.24 ± 0.02	24.22 ± 1.61	26.48 ± 0.06	25.83 ± 0.09	*0.0979*
PUFA	40.38 ± 0.01 a	42.11 ± 0.39 b	42.57 ± 0.41 b	40.23 ± 0.37 a	41.51 ± 0.02 ab	*0.0019*
Others	5.07 ± 0.12 b	5.12 ± 0.33 b	3.60 ± 0.52 a	5.52 ± 0.24 b	4.73 ± 0.03 ab	*0.0009*
n3	21.71 ± 0.08 a	22.69 ± 0.11 b	23.39 ± 0.22c	21.76 ± 0.27 a	22.55 ± 0.01 b	*0.0007*
n6	18.67 ± 0.09 a	19.42 ± 0.28c	19.19 ± 0.19c	18.47 ± 0.15 a	18.96 ± 0.03 b	*0.0115*
n9	20.92 ± 0.05	19.73 ± 0.01	18.83 ± 1.61	20.65 ± 0.03	20.36 ± 0.069	*0.1553*
n3/n6	1.16 ± 0.01 a	1.17 ± 0.01 a	1.22 ± 0.01c	1.18 ± 0.01 a	1.19 ± 0.01 b	*0.0044*
EPA/DHA	0.29 ± 0.01 b	0.28 ± 0.01 ab	0.26 ± 0.00 a	0.28 ± 0.01 aab	0.27 ± 0.02 a	*0.0157*

Values are expressed as mean ± SD (n = 6). Dietary codes: Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

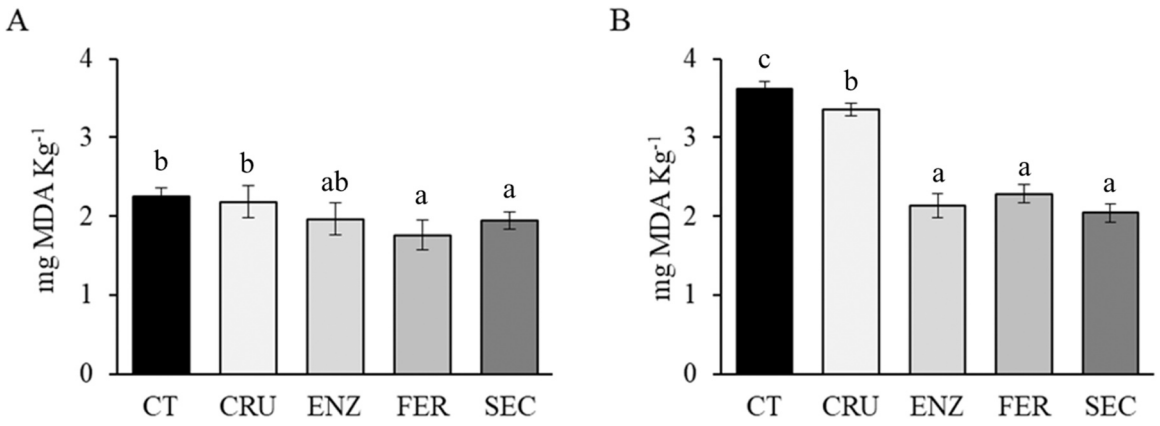

Fig. 2. TBARS content of muscle (A) and liver (B) samples of seabass juveniles at the end of the feeding trial (n = 6). Dietary codes: Dietary codes: CT (Control diet); CRU (5% crude biomass of *R. okamurae*); ENZ (5% hydrolysed *R. okamurae* biomass); FER (5% fermented *R. okamurae* biomass); Section (5% sequential hydrolysis and fermented *R. okamurae* biomass). Different lowercase letters in the line indicate significant differences among the experimental groups ($p < 0.05$).

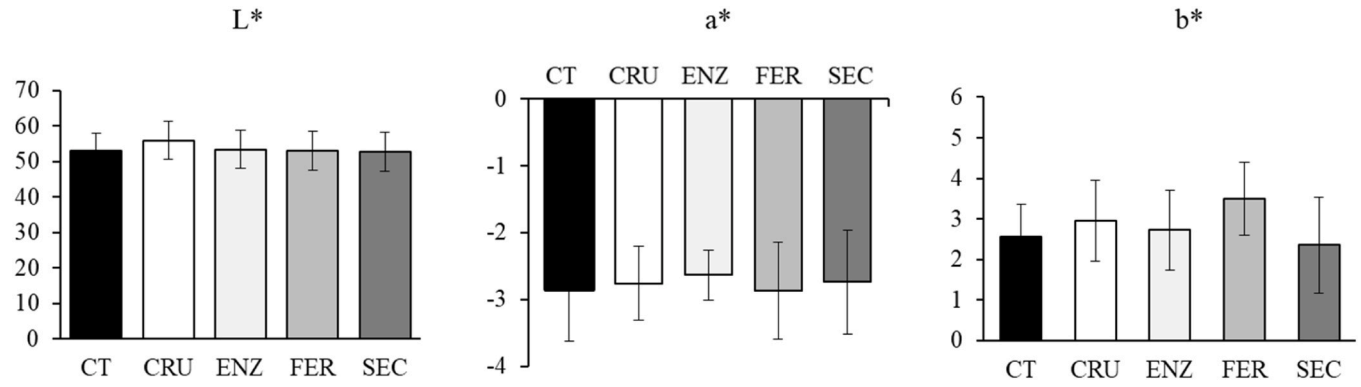

Fig. 3. Skin color parameters (L*, a*, b* according to CIE (1986)) in juvenile European seabass, *D. labrax,* at the end of the feeding trial. Superscript letters indicate significant difference between experimental diets ($p < 0.05$). Color parameters L*, a* and b* stand for lightness, redness, and yellowness, respectively, according to Commission Internationale de l'Eclairage (CIE, 1986).

Table 5
Blood and hepatic biochemistry of juvenile European sea bass at the end of the feeding trial.

	CT	CRU	ENZ	FER	SEC	*p*
Plasma glucose (mM)	7.85 ± 0.71	6.58 ± 0.49	7.22 ± 0.79	6.30 ± 0.37	5.94 ± 0.56	*0.193*
Plasma lactate (mM)	2.47 ± 0.12^{a}	3.11 ± 0.08^{b}	2.87 ± 0.09^{b}	2.65 ± 0.14^{ab}	2.34 ± 0.17^{a}	*< 0.001*
Plasma triglycerides (mM)	16.8 ± 1.50^{a}	12.0 ± 1.34^{b}	14.8 ± 0.65^{ab}	14.1 ± 0.53^{ab}	16.1 ± 0.76^{ab}	*0.019*
Plasma cholesterol (mg dL^{-1})	235.8 ± 14.6^{a}	136.8 ± 5.6^{b}	204.4 ± 5.6^{a}	203.2 ± 11.4^{a}	227.6 ± 14.6^{a}	*< 0.001*
Plasma proteins (mg mL^{-1})	27.5 ± 0.96	26.8 ± 1.27	28.5 ± 0.49	28.1 ± 1.08	27.4 ± 1.24	*0.815*
Plasma cortisol (ng mL^{-1})	33.3 ± 6.22^{a}	24.1 ± 5.68^{ab}	14.6 ± 3.69^{ab}	12.1 ± 2.93^{b}	8.3 ± 2.44^{b}	*0.011*
Hepatic glucose (μmol·gww^{-1})	3.93 ± 0.19	3.98 ± 0.25	3.87 ± 0.19	4.03 ± 0.20	4.07 ± 0.18	*0.962*
Hepatic glycogen (μmol·gww^{-1})	19.1 ± 0.78	20.4 ± 0.84	18.4 ± 0.76	20.4 ± 1.10	20.3 ± 1.12	*0.423*
Hepatic triglycerides (μmol·gww^{-1})	64.8 ± 5.22^{ab}	75.3 ± 4.73^{a}	61.0 ± 4.78^{ab}	64.1 ± 3.18^{ab}	56.7 ± 3.04^{b}	*0.049*

Data are the mean ± SD of 24 fish/experimental group. Dietary codes: Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

The profile of the intestinal protease fractions does not seem to be influenced by the inclusion of *R. okamurae* biomass, as all animals showed the same number and distribution of active fractions as fish fed with CT diet.

3.8. Histological evaluation of liver and intestine

The histological characteristics of liver sections from fish fed the different dietary treatments are shown in Fig. 5. Overall, all fish exhibited hepatocytes of normal shape and regular morphology, without evidence of necrosis or steatosis. However, moderate vacuolization in fish fed on CT, CRU and ENZ diets was observed. The morphological analysis of the images obtained by light microscopy revealed that fish fed on FER and SEC diets showed hepatocytes smaller than those of fish fed on the other dietary treatments (Table 8).

Fig. 6 shows the intestinal sections from juvenile seabass at three magnification levels (40x, 100x and 400x). In general, no signs of intestinal damage were found, as all the specimens presented intestinal mucosa without evidence of abnormalities. The morphometric analysis (Table 9) revealed that the height of the enterocytes was similar in all treatments except in fish fed with fermented algae (FER), which were significantly lower ($p < 0.05$). Additionally, the thickness of the serosa,

Table 6
Metabolic enzymes in liver of juvenile European sea bass at the end of the feeding trial.

	CT	CRU	ENZ	FER	SEC
HOAD (U/mg prot)	1,48 ± 0,41 a	2,34 ± 0,64 b	1,84 ± 0,64 ab	1,96 ± 0,42 ab	2,06 ± 0,70 ab
HK (U/mg prot)	33,08 ± 15,41 ab	44,03 ± 13,09 b	28,04 ± 14,90 a	30,67 ± 8,40 a	25,91 ± 6,50 a
LDH (mU/mg prot)	221,85 ± 18,96	264,34 ± 49,42	241,39 ± 35,62	210,92 ± 30,69	222,99 ± 26,32
FBP (U/mg prot)	1,85 ± 0,26	1,98 ± 0,52	1,92 ± 0,42	1,85 ± 0,29	1,84 ± 0,32
GPact (U/mg prot)	6,02 ± 2,08	6,34 ± 4,24	5,98 ± 0,47	5,98 ± 3,60	6,23 ± 1,35
PK (U/mg prot)	93,13 ± 9,38	94,84 ± 13,33	93,39 ± 5,72	93,25 ± 5,24	93,93 ± 13,52

Values are expressed as mean ± SD (n = 12). Dietary codes: Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). HOAD: 3-hydroxyacyl-CoA dehydrogenase; HK: hexokinase; LDH: lactate dehydrogenase; FBP: fructose 1,6-bisphosphatase; GPact glycogen phosphorylase active; PK: pyruvate kinase. Values in the same row with different lowercase letters indicate significant differences among the experimental groups ($p < 0.05$).

muscular and submucosa layers, as well as the *lamina propria*, was significantly lower in fish fed on diets that included the pre-treated algal biomass, especially the fermented (FER) and the sequentially hydrolysed and fermented (SEC) biomasses.

3.9. Ultrastructural analysis of the intestinal mucosa

TEM and SEM (Fig. 7) observations confirmed that none of the dietary treatments damaged the brush border integrity of enterocytes. All the experimental groups showed an intestinal mucosa with normal appearance, with a well-defined and organised brush border membrane. Morphometric analysis of TEM and SEM images revealed significant differences in microvilli length, microvilli diameter, enterocyte apical area and enterocyte absorption surface among the different dietary treatments evaluated (Table 10). Overall, the dietary inclusion of pre-treated *R. okamurae* (ENZ, FER and SEC diets) induced a significant increase ($p < 0.05$) in microvilli length compared with fish fed on the algae-free diet. The enterocyte apical area was also higher in fish fed *R. okamurae* ($p < 0.05$). Finally, a significant increase in the total absorption surface per enterocyte was observed in fish fed with *R. okamurae* compared to the values obtained in fish fed on CT diet, especially in ENZ and SEC groups.

4. Discussion

This study evaluates the potential of *Ruguloteryx okamurae* biomass for feeding seabass juveniles. To date, no previous studies evaluating the inclusion of raw or pre-treated of *R. okamurae* biomass in aquaculture feeds are available, with the exception of the recently published work by Fonseca et al. (2023). *Ruguloteryx okamurae* presents a high molecular diversity and certain interest from a nutritional perspective, i.e high level of carbon compounds (35% total internal carbon), high level of lipids (8%) compared to other macroalgae, as well as polyphenols with antioxidant capacity (Vega et al., 2023). In general, the effects of including macroalgae in fish feeds on fish physiology appear to be species-specific and dependent on the inclusion level. Previous studies reported that some macroalgae can be used as a dietary ingredient in different farmed fish species, such as gilthead seabream (*Sparus aurata*) (Vizcaíno et al., 2019; Molina-Roque et al., 2022) or Senegalese sole (*Solea senegalensis*) (Sáez et al., 2020), pointing to promising results in terms of growth, survival and nutrient utilization. However, despite the low inclusion level tested in this study, fish fed on 5% crude algal biomass (CRU), as well as those fed on hydrolysed (ENZ) and fermented (FER) biomass reached lower final body weight than fish fed on algae-free diet (CT). This detrimental impact of fish growth might be related to the presence of a high content of soluble and insoluble polysaccharides in *R. okamurae* biomass that might well have increased transit of feed through the digestive tract of fish, which can even impair the specific growth rate (Vizcaíno et al., 2016). In addition, *R. okamurae* from the southern coasts of Spain contains a wide range of secondary metabolites of the diterpenoid class, among which dilkamural is the most abundant (Cuevas et al., 2021, 2023). Moreover, this compound seems to be responsible for the invasive capacity of the alga, given its deterrent capacity and toxic effects on generalist herbivores such as the sea urchin *Paracentrotus lividus* (Casal-Porras et al., 2021). However, unlike fresh, wet *R. okamurae*, the dried raw algal biomass used in this

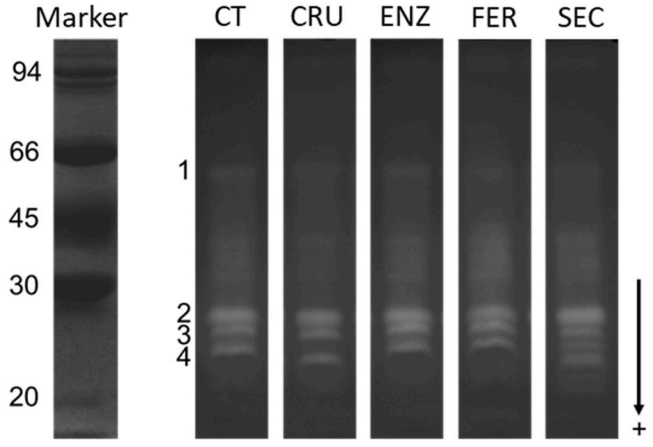

Fig. 4. Zymograms of alkaline proteolytic activity in the intestine extracts of juvenile *D. labrax* fed on the experimental diets. Dietary codes: CT (Control diet); CRU (5% crude biomass of *R. okamurae*); ENZ (5% hydrolysed *R. okamurae* biomass); FER (5% fermented *R. okamurae* biomass); Section (5% sequential hydrolysis and fermented *R. okamurae* biomass). Protein standards employed were phosphorylase b (94 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa). Five microliters of molecular weight marker (M) were loaded.

Table 7
Digestive enzyme activities (U g tissue^{-1}) measured in intestinal extracts of juvenile *D. labrax* at the end of the feeding trial.

	CT	CRU	ENZ	FER	SEC
Leucine aminopeptidase	0.36 ± 0.05 b	0.27 ± 0.03 a	0.30 ± 0.07 ab	0.30 ± 0.02 ab	0.30 ± 0.06 ab
Alkaline phosphatase	3.53 ± 0.46c	2.56 ± 0.34 a	2.93 ± 0.19 ab	3.33 ± 0.31 bc	3.03 ± 0.52c
Trypsin	0.02 ± 0.01 a	0.03 ± 0.01 ab	0.03 ± 0.01 ab	0.04 ± 0.01 b	0.03 ± 0.01 ab
Chymotrypsin	0.50 ± 0.07 a	0.48 ± 0.08 a	0.64 ± 0.11 b	0.55 ± 0.05 ab	0.56 ± 0.05 ab
Alkaline protease	168.71 ± 29.46 a	184.09 ± 12.58 a	284.85 ± 39.60c	235.33 ± 36.68 bc	228.88 ± 16.83 b
p	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

Values are expressed as mean ± SD (n = 6). Dietary codes: Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). Values in the same row with different lowercase letters indicate significant differences among the experimental groups ($p < 0.05$).

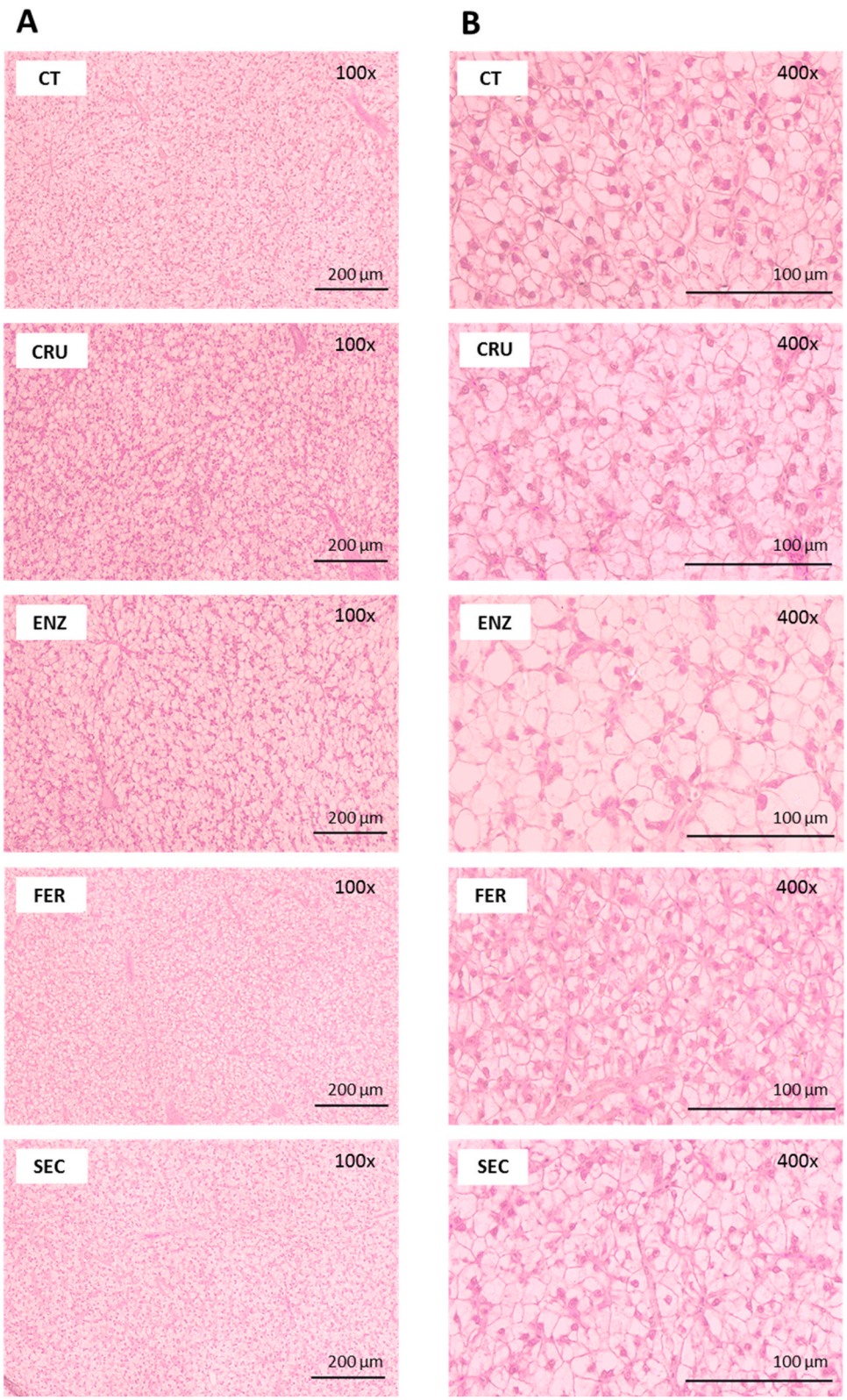

Fig. 5. Liver sections of juvenile *D. labrax* fed on the experimental diets. Dietary codes: CT (Control diet); CRU (5% crude biomass of *R. okamurae*); ENZ (5% hydrolysed *R. okamurae* biomass); FER (5% fermented *R. okamurae* biomass); Section (5% sequential hydrolysis and fermented *R. okamurae* biomass). H&E stain, magnification 100x (A) and 400x (B).

study contained, instead of dilkamural, small amounts of a transformation product, 2-deacetoxy-2,3-didehydrodilkamural (DKE; Fig. S1), this suggesting that drying of the algal biomass prior to its use promote the transformation and degradation of dilkamural. Therefore, data suggest that, eventually, the transformation product DKE might be involved in the negative effects of feeds containing crude algal biomass. The compound DKE was still present in algal biomass after enzymatic hydrolysis or fermentation, although at significantly lower

Table 8
Quantification of the histological parameters assessed in the intestine of juvenile *D. labrax* at the end of feeding trial.

	Hepatocyte area (μm^2)	Hepatocyte major axis (μm)
CT	115.82 ± 15.04 b	16.18 ± 1.76c
CRU	114.52 ± 18.85 b	15.93 ± 2.56c
ENZ	112.80 ± 25.76 b	16.17 ± 2.00c
FER	99.50 ± 22.24 a	12.61 ± 1.74 b
SEC	95.40 ± 12.61 a	11.64 ± 1.40 a
p	*0.015*	*0.009*

Values are mean ± SD (n = 50). Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). Values in the same column with different lowercase letters indicate significant differences among the experimental groups ($p < 0.05$).

concentrations than in crude biomass, and it was not observed in algal biomass derived from sequential hydrolysis and fermentation. These results are consistent with the lack of negative effects in fish fed with the diet that included the sequentially pre-treated biomass (SEC), whose final body weight values were similar to those observed in fish fed the control diet (CT). This finding supports the effectiveness of the sequential treatment of enzymatic hydrolysis plus fermentation in order to improve the feasibility of this algal biomass as dietary ingredient for aquafeeds, although additional and more specific research on the chemical composition of the pre-treated biomass is needed to confirm this hypothesis.

Regarding muscle proximal composition, the dietary inclusion of *R. okamurae* increased fish protein content, especially in animals fed on hydrolysed (ENZ) and sequentially treated (SEC) algae. This effect has also been described in previous studies on different macroalgal meals, where the increase has been attributed to the high protein content of the biomass, but this does not seem to be the case in the present study, given that the experimental feeds are isoproteic (Table 1), and in addition, soluble protein content is low in *R. okamurae* (Vega et al., 2023).

Other authors have related the high content in ascorbic acid observed in some species of macroalgae to increased lipid metabolism in fish, which spares protein for growth and tissue development (Monier *et al.*, 2022). Although no quantitative differences in total lipid content were observed in this study (Table 3), qualitative differences were found in the fatty acid profile of muscle (Table 4), which may provide relevant information on the influence of feed on the chemical composition of fish (Fernandes et al., 2012). While dietary fatty acid profile is usually reflected in fish muscle, the significant increase in total PUFAs, especially in ARA and DHA content, observed in fish fed on algae-supplemented diets cannot be attributed to differences in dietary fatty acids owing to *R. okamurae* inclusion. This apparent selective retention has been reported in previous studies, and it seems to be related to a preferential retention of ARA and DHA during the biosynthesis and remodelling of phospholipids (Bell et al., 2002; Tocher, 2010). Furthermore, this finding could also be attributed to the effects of dietary fatty acid composition on gene expression and activity of key enzymes involved in lipid metabolism, which ultimately determine the accumulation of specific fatty acids in fish tissues (Peng et al., 2014).

Macroalgae are known to contain a wide array of bioactive compounds of potential interest for aquaculture (Sáez et al., 2020), standing out substances with antioxidant properties like vitamins, carotenoids and polyphenols. According to Remya et al. (2022), brown algae have relatively higher levels of powerful antioxidants than green and red algae. Among them, a particular group is that of brown algal polyphenols, also called phlorotannins. These compounds have strong antioxidant activities against free radical mediated oxidation damage (Li et al., 2011). Therefore, the presence of this kind of antioxidant substances could explain the prevention of lipid oxidation is muscle and liver tissue observed in this study (Fig. 2). Beside this, the implementation of the different biotechnological pre-treatments seems to increase the antioxidant effects on muscle and liver lipids of algae biomass, likely owing to increased release of the inner antioxidant substances (Sáez et al., 2022; Galafat et al., 2020).

Additionally, macroalgae are a natural source of a wide variety of pigments (Sáez et al., 2020). In particular, fucoxanthin is dominant in brown seaweeds, overshadowing other pigments like chlorophyll and other carotenoids (Kumar et al., 2008). This pigment is responsible for its brown to yellow hue and possesses antioxidant activity (Din et al., 2022). In addition to the antioxidant and anti-inflammatory effects (Liu et al., 2020), the presence of pigments in algae has been closely linked to effects on fish pigmentation. Indeed, previous studies have confirmed that dietary macroalgal supplementation can exert a favourable impact on fish colour attributes, improving lightness (L*), yellowness (b*) and redness (a*) (Moroney et al., 2015; Sáez et al., 2020), although these effects have not been observed in the present work.

Regarding to the metabolic reorganization, lower level of TAG and cholesterol were observed in fish that ingested CRU diet compared to the other diets, indicating an certain effect on lipid metabolism, in agreement with previous studies evaluating plant protein in fish diets (Dias et al., 2005; Messina et al., 2013). CRU diet also led to high level of lactate, as well as ENZ diet, suggesting that the use of these diets promote a higher anaerobic metabolism that, in turn, might be due to a lower availability of lipids for oxidation. The lower level of circulating TAG might support this hypothesis. These findings strongly correlate with key steps of fatty acid oxidation (HOAD) and lactate production (LDH), suggesting that crude extracts may promote these metabolic enzymes to help the entry of fatty acids in the mitochondria and their oxidative capacity, as well as the hepatic uptake of lactate to compensate energy expenditure in fish fed the CRU diet, as suggested in previous studies incorporating algae or other vegetal sources in aquafeeds (Pérez-Jiménez et al., 2012; Perera et al., 2020; Molina-Roque et al., 2022). The increase in the enzymatic activity of HK, the first step in glycolytic pathway by phosphorylating glucose to be easily wearable by cells, also points to an extra investment to promote the metabolic use of carbohydrate as energy source in the liver. On the other hand, sustained high level of cortisol in fish is known to negatively affect growth rate, due to higher energy expenditure along with changes in amino acid catabolism, higher glycogen use, and storage of lipids in the liver (Jerez-Cepa et al., 2019). Conversely, when cortisol level are low, glycogen reserves, protein synthesis and growth are restored (Van Der Boon et al., 1991; Milligan, 2003). In our study, cortisol level decreased as the processing of algal biomass increased, with higher level in the control diet without algae. Although cortisol levels measured in all the experimental groups were within the normal range in unstressed fish, the lower levels in fed fish ENZ+FER support our observations growth-promoting changes in lipid metabolism. It might well be hypothesized that the lower cortisol levels in these fish allow them to cope better with stressful situations without major changes in growth. This specific topic will be assessed in further studies, in which sea bass fed biotechnologically treated algae (e.g. ENZ+FER) deal with different stress sources, such as emersion, transport or high farming density.

From a nutritional point of view, the sustainable use of *R. okamurae* as an aquafeed ingredient, even at low levels, has some drawbacks that may have a direct impact on fish digestive functionality. As it was mentioned above, the carbohydrate composition of brown macroalgae is complex and it includes the cell-wall polysaccharides, alginate and fucoidan, and the storage polysaccharide laminarin and mannitol (Groisillier et al., 2014). In addition *R. okamurae* also contains other compounds, such as dilkamural or its transformation products that might cause adverse effects on fish digestive functionality.

In this regard, the evaluation of digestive enzymes and the knowledge of their role in the digestion and absorption processes can be used as a suitable predictor of potential feed utilisation, growth differences and health status in fish (Zhang et al., 2018; García-Márquez et al., 2023). It is widely known that fish can adapt their metabolic functions to

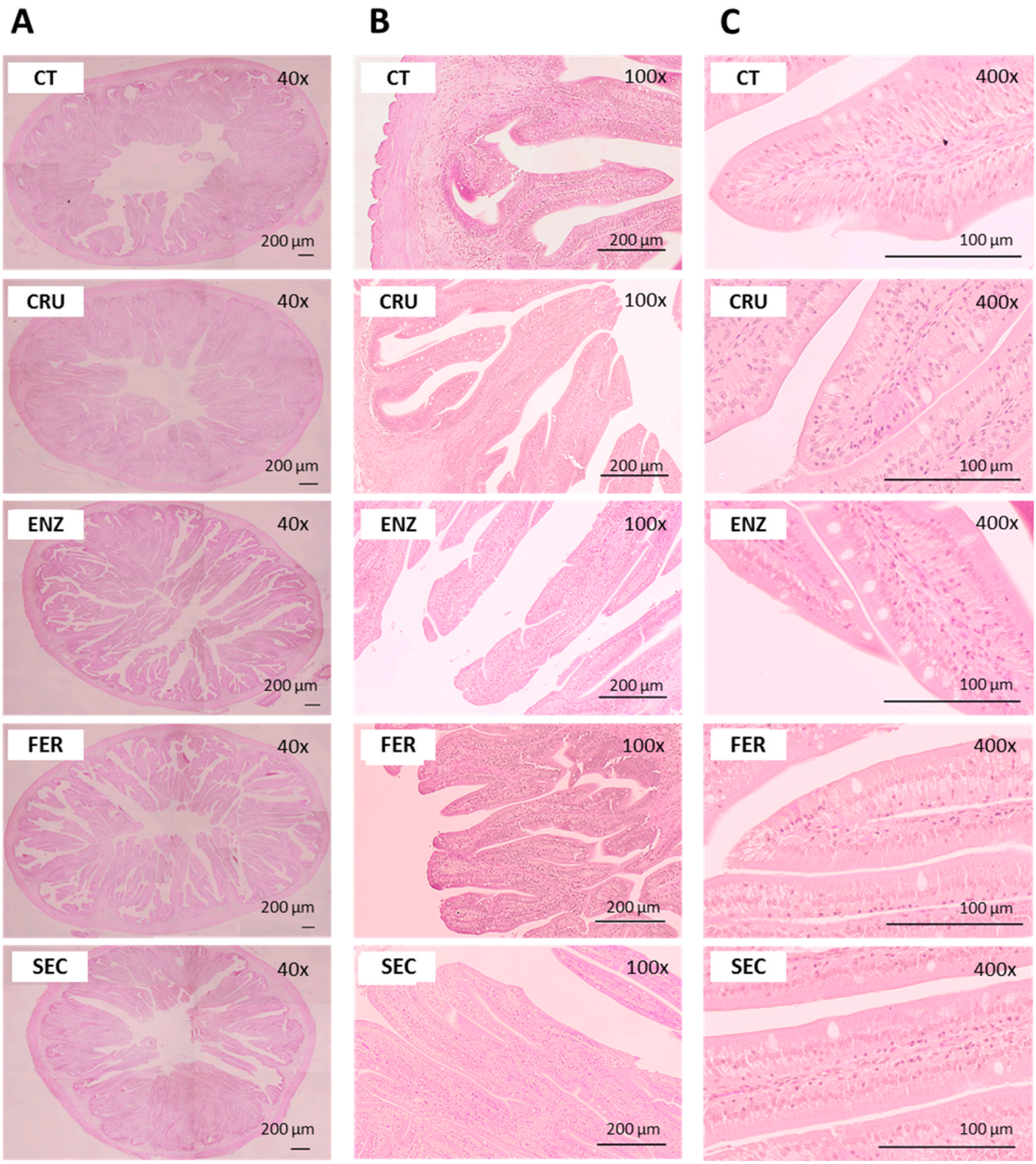

Fig. 6. Cross section of intestines of juvenile *D. labrax* fed on the experimental diets. Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed *R. okamurae* biomass); FER (5% fermented *R. okamurae* biomass); Section (5% sequential hydrolysis and fermented *R. okamurae* biomass). H&E stain, magnification 40x (A), 100x (B) and 400x (C).

the dietary substrates, through a regulation in enzyme secretion, in order to improve the utilisation of feed ingredients (Caruso et al., 2019). These adaptive responses occur in a short time and they are useful for assessing the impact of non-conventional ingredients on the intestinal function (Silva et al., 2010).

In this study, the inclusion of *R. okamurae* exerted a significant effect on the activity levels of the different enzymes evaluated. A similar effect was observed for both pancreatic and brush border digestive enzymes. In agreement with the findings observed for fish growth, fish fed with 5% crude biomass of *R. okamurae* (CRU) showed a significant decrease in the activity of digestive enzyme, although this negative effect was partially, or almost completely reversed when the algal biomass was pre-treated, yielding then values similar to those observed in the control group (or even higher), especially in the case of the pancreatic secretion enzymes,

Table 9
Quantification of the histological parameters assessed in the intestine of juvenile *D. labrax* at the end of the feeding trial.

	CT	CRU	ENZ	FER	SEC
EH (μm)	34.2 ± 4.66 b	36.89 ± 3.43 b	34.93 ± 4.54 b	29.72 ± 3.17 a	33.17 ± 3.94 b
SL (μm)	32.79 ± 5.17 b	37.96 ± 5.40c	30.77 ± 4.20 ab	28.96 ± 5.07 a	29.34 ± 262 a
mL (μm)	48.14 ± 8.47 cd	50.06 ± 7.95 d	34.30 ± 7.97 a	44.02 ± 9.43 bc	40.66 ± 8.96 b
SML (μm)	33.48 ± 4.94 d	49.47 ± 7.08 e	28.58 ± 2.84c	26.21 ± 3.62 ab	23.86 ± 3.03 a
LP (μm)	11.87 ± 1.51c	11.7 ± 0.69c	10.8 ± 1.02 b	10.44 ± 0.57 b	9.55 ± 0.55 a
p	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

Values are mean ± SD (n = 50). Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). Values in the same row with different lowercase letters indicate significant differences among the experimental groups ($p < 0.05$). Abbreviations stand for Enterocyte height (EH, μm), Serosa layer (SL, μm), Muscular layer (mL, μm), Submucosa layer (SML, μm), lamina propria (LP, μm).

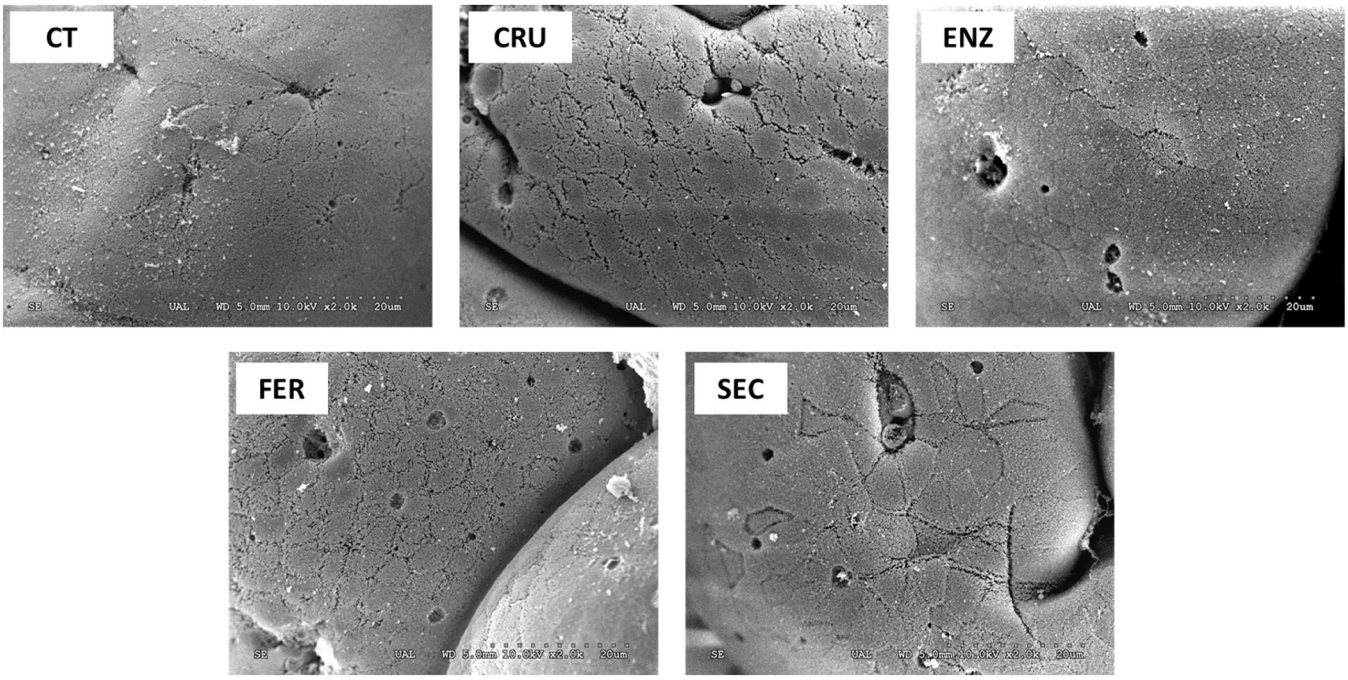

Fig. 7. Comparative SEM micrographs from the intestine of juvenile *D. labrax* at the end of the feeding trial. Dietary codes: CT (Control diet); CRU (5% crude biomass of *R. okamurae*); ENZ (5% hydrolysed *R. okamurae* biomass); FER (5% fermented *R. okamurae* biomass); Section (5% sequential hydrolysis and fermented *R. okamurae* biomass). Scale bar: 20 μm.

Table 10
Microvillar morphology of the intestine of juvenile European seabass at the end of the feeding trial.

	mL (μm)	MD (μm)	EA (μm)	TAS (μm^2)
CT	1.80 ± 0.16 a	0.11 ± 0.01	20.07 ± 2.47 a	641.69 ± 65.59 a
CRU	2.13 ± 0,.33 bc	0.10 ± 0.01	22.74 ± 2.85c	869.73 ± 87.20 b
ENZ	2.39 ± 0.30c	0.11 ± 0.01	20.84 ± 2.31 ab	1001.22 ± 98.15c
FER	2.05 ± 0.19 b	0.11 ± 0.01	22.10 ± 1.97 bc	925.26 ± 82.35 b
SEC	2.36 ± 0.24c	0.11 ± 0.01	26.15 ± 2.99 d	1113.66 ± 116.92 d
p	*< 0001*	*0650*	*< 0001*	*< 0001*

Values are mean ± SD (n = 50). Dietary codes: CT (Control diet); CRU (5% crude biomass of R. okamurae); ENZ (5% hydrolysed R. okamurae biomass); FER (5% fermented R. okamurae biomass); Section (5% sequential hydrolysis and fermented R. okamurae biomass). mL: microvilli length; MD: microvilli diameter; EA: enterocyte apical area; TAS: enterocyte absorption surface. Values in the same column with different lowercase letters indicate significant differences among the experimental groups ($p < 0.05$).

which play a crucial role in feed hydrolysis (Vizcaíno et al., 2014). This fact could be related to increased availability of small peptides and free amino acids in the pre-treated algal biomasses that may stimulate the enzyme secretion (Zambonino-Infante et al., 2008). On the other hand, enzymatic hydrolysis could also have the potential to enhance nutrient bioavailability by breaking the glycosidic linkages between sugar monomers that form the indigestible polysaccharide algal cell wall, as well as to inactivate antinutritive factors (Vizcaíno et al., 2020). In fact, the processing of land-based crops, such as soybean or lupin, through the application of exogenous enzymes has overcome the effects of anti-nutritional factors and digestibility issues, enabling the use of high dietary inclusion levels of these ingredients for less tolerant fish species (Wan et al., 2019). Similarly, previous studies also pointed out that microbial fermentation processes on different raw materials, such as plant protein ingredients, can remove or reduce the presence of anti-nutritive factors that, otherwise, would inhibit the activity of digestive enzymes, thus increasing the bioavailability of nutrients (Mugwanya *et al.*, 2022). Although the treatments applied to the algal biomass of *R. okamurae* were primarily selected and designed for improving the digestibility and nutrient availability, they also affected the content of dilkamural, a potentially harmful secondary metabolite of the alga. In this regard, recent reports describe the ability of enzymatic and microorganism-based strategies for the control and removal of toxic secondary metabolites, especially of fungal origin, from feeds, (Nesic et al., 2021). Nonetheless, studies to assess the effects of dilkamural and other secondary metabolites of *R. okamurae*, as well as of their

transformation products on the physiology of fish would be needed, in order to define the more suitable strategy address and control the presence of these compounds in the feeds.

As mentioned above, liver histology revealed the lack of major alterations in the structure and morphology in any of the experimental groups evaluated. Only moderate vacuolization of the hepatocytes in some of the samples evaluated was observed, in agreement with the study by Vizcaíno et al. (2019). They found a slight vacuolization of hepatocytes in specimens of Senegale sole (*Solea senegalensis*) fed on 5% *Ulva*-supplemented diets for 90 days, which could be explained by the unrestricted feeding and the natural disposition of fish like seabass to deposit lipid in the liver (Haas et al., 2016).

On the other hand, a healthy gut is essential for optimal animal performance (Sweetman et al., 2008). Any alteration in the integrity of this barrier can activate a strong response of immune cells and lead to chronic inflammation of the intestinal tissues that hinders the adequate nutrient absorption of nutrients and the efficient transfer of nutrients to the blood, as well as its role as a physical barrier against pathogenic microorganisms (Sweetman et al., 2008; Vizcaíno et al., 2014). Any negative effect on the intestinal mucosa could lead to inefficient feed conversion, as well as to increased energy consumption for directing valuable resources from growth to the more immediate urgency of tissue repair and maintenance (Sweetman et al., 2008). There is evidence confirming that the inclusion of macroalgae in aquafeeds appears to be associated with a positive effect on gut morphology in different fish species like gilthead seabream (*Sparus aurata*) or Senegalese sole (*Solea senegalinsis*) (Vizcaíno et al., 2016, 2019, respectively). Regarding the results obtained in this study, no negative effects or damage to the apical brush border were observed that could be attributable to the dietary inclusion of *R. okamurae* biomass in the diet, and even some positive changes in the structure of the villi and the microvilli were observed. Both the enterocyte height, as well as the thickness of the *lamina propria*, and the serosa, muscular and submucosa layers revealed a marked reduction in fish fed with the pre-treated biomass of *R. okamurae*, in comparison with CRU (fed with raw biomass) and control (CT) groups. This findings are likely attributable to the lack of inflammatory processes or accumulation of lipid vacuoles inside the enterocytes, which are compatible with the processes of enteritis and/or intestinal steatosis (Urán et al., 2008). This reduction in the thickness of the l*amina propria* and the serous, muscular, and submucosal layers was especially evident in the fish fed with diets including the fermented algae (FER and SEC), which might be related to the presence of specific microorganisms derived from the fermentation processes. In fact, some authors (Batista et al., 2016) suggest the influence of the incorporation of probiotic microorganisms on the morphology of the intestine.

Similarly, the electron microscopy analysis revealed positive effects of the processed algal biomass on the morphology of fish intestinal mucosa. The results revealed a significant increase in both microvilli length and enterocyte apical area, resulting in a significant increase in the absorptive surface area per enterocyte and, thus, a possible increase in nutrient absorption capacity, as well as an improvement in the contribution of the intestinal mucosa as a physical barrier compared to that observed in fish fed the free-algae diet (Vizcaíno et al., 2014).

In conclusion, the results obtained in this work confirm that the biomass of the invasive algae *R. okamurae* may be suitable for use as a dietary ingredient for feeding European seabass juveniles. Likewise, this piece of research reveals the effectiveness of novel and low-cost biotechnological treatments, based on enzymatic hydrolysis and fermentation, as a tool for the recovery and valorisation of the biomass of this invasive alga that could be used as a potential solution for the mititation of the environmental problems generated. However, future studies focused on the intrinsic mechanisms of its effects, as well as in the in-depth knowledge of this invasive species are needed.

Author statement

The authors confirm sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

CRediT authorship contribution statement

Antonio J. Vizcaíno: Designed the research, Data curation, Writing the original draft, Review & editing. **María Isabel Sáez, Alba Galafat, Rubén Galindo-Melero and Erick Perera**: Performed experiments, Data curation, Review**. Isabel Casal-Porras and Eva Zubía:** Investigation, Review & editing. **Julia Vega**: Resources and review. **Juan Antonio Martos-Sitcha**: Performed experiments, Funding acquisition, Data curation, Review. **Félix Figueroa**: Resources and review. **Tomás Francisco Martínez**: Funding acquisition, Review & editing**; Francisco Javier Alarcón**: Funding acquisition, Designed the research, Review & editing. All authors agreed with the final submitted version. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by the Projects VALINVA (Proyecto de Innovación Empresarial con Proyección Territorial en el Ámbito de la Economía Azul CEI·MAR (CEIMAR 2019), the Project RUGULOFEED (PCM_00081) (Programa de Ayudas a Proyectos de I+D+i, en régimen de concurrencia competitiva, en el marco del Plan Complementario de Ciencias Marinas y del Plan de Recuperación, Transformación y Resiliencia, Junta de Andalucía) and the Spin-off LifeBioencapsulation S.L. Authors also thank the contribution of the projects BLUEMARO (PID2020-116136RB-I00, Ministerio de Ciencia e Innovación, Spain), PPUENTE2022/007 (Plan Propio de Investigación y Transferencia de la Universidad de Almería), AQUATECH4FEED (grant # PCI2020-112204) granted by MCIN/AEI/10.13039/501100011033 and the EU "NextGenerationEU"/PRTR within the ERA-NET BioBlue COFUND, NEOGIANT (grant # 101036768) granted by the European Union's Horizon 2020 research and innovation programme. A. Galafat received a Margarita Salas postdoc grant (RR_A_2022_03, Ministerio de Universidades, Spain).

All the experimental diets were elaborated by the Universidad de Almería (Experimental feeds Service; http://www.ual.es/stecnicos_spe). Experimental Feeds Service was granted by EQC2018–004984-P and EQC2019–006380-P.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101877.

References

Alarcón, F.J., Díaz, M., Moyano, F.J., Abellán, E., 1998. Characterization and functional properties of digestive proteases in two sparids; gilthead sea bream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish. Physiol. Biochem. 19, 257–267. https://doi.org/10.1023/A:1007717708491.

AOAC, 2002. Official Methods of Analysis of AOAC International. 17th edition. 1st revision. Gaithersburg, MD, USA, Association of Official Analytical Communities (2002).

Aslamyah, S., Karim, M.Y., Badraeni, B., 2017. Fermentation of seaweed flour with various fermentors to improve the quality of fish feed ingredients. JAI 16 (1), 8–14. https://doi.org/10.19027/jai.16.1.8-14.

Batista, S., Medina, A., Pires, M.A., Moriñigo, M.A., Sansuwan, K., Fernandes, J.M.O., Valente, L.M.P., Ozório, R.O.A., 2016. Innate immune response, intestinal morphology and microbiota changes in Senegalese sole fed plant protein diets with probiotics or autolysed yeast. Appl. Microbiol. Biotechnol. 100 (16), 7223–7238. https://doi.org/10.1007/s00253-016-7592-7.

Bell, J.G., Henderson, R.J., Tocher, D.R., McGhee, F., Dick, J.R., Porter, A., Smullen, R.P., Sargent, J.R., 2002. Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. J. Nutr. 132 (2), 222–230. https://doi.org/10.1093/jn/132.2.222.

Bergmeyer, H.V., 1974. Phosphatases Methods of Enzymatic Analysis, vol. 2. Academic Press, New York.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. In: Methods in enzymology, vol. Vol. 52. Academic Press, pp. 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6.

Caruso, M.C., Galgano, F., Grippo, A., Condelli, N., Di Cairano, M., Tolve, R., 2019. Assay of healthful properties of wild blackberry and elderberry fruits grown in Mediterranean area. J. Food Meas. Charac. 13 (2), 1591–1598. https://doi.org/10.1007/s11694-019-00075-x.

Casal-Porras, I., Zubía, E., Brun, F.G., 2021. Dilkamural: a novel chemical weapon involved in the invasive capacity of the alga *Rugulopteryx okamurae* in the Strait of Gibraltar. Estuar. Coast. Shelf Sci. 257, 107398 https://doi.org/10.1016/j.ecss.2021.107398.

Chen, J., Li, H., Zhao, Z., Xia, X., Li, B., Zhang, J., Yan, X., 2018. Diterpenes from the marine algae of the genus Dictyota. Mar. Drugs 16, 159. https://doi.org/10.3390/md16050159.

CIE. 1978. International commission on illumination, recommendations on uniform colour spaces, colour difference equations, psychometric colour terms. CIE Publication No. 15 (E-1.3. 1) 1971/(TO-1.3).

Cuevas, B., Arroba, A.I., De los Reyes, C., Gómez-Jaramillo, L., González-Montelongo, M. C., Zubía, E., 2021. Diterpenoids from the brown alga *Rugulopteryx okamurae* and their anti-inflammatory activity. Mar. Drugs 19, 677. https://doi.org/10.3390/md19120677.

Cuevas, B., Arroba, A.I., De los Reyes, C., Zubía, E., 2023. *Rugulopteryx*-derived spatane, secospatane, prenylcubebane and prenylkelsoane diterpenoids as inhibitors of nitric oxide production. Mar. Drugs 21, 252. https://doi.org/10.3390/md21040252.

De Paula, J., Vallim, M., Teixeira, V., 2011. What are and where are the bioactive terpenoids metabolites from *Dictyotaceae* (*Phaeophyceae*). Rev. Bras. Farmacogn. 21, 216–228. https://doi.org/10.1590/S0102-695X2011005000079.

DelMar, E.G., Largman, C., Broderick, J.W., Geokas, M.C., 1979. A sensitive new substrate for chymotrypsin. Anal. Biochem. 99, 316–320. https://doi.org/10.1016/S0003-2697(79)80013-5.

Demarco, M., de Moraes, J.O., Matos, Â.P., Derner, R.B., de Farias Neves, F., Tribuzi, G., 2022. Digestibility, bioaccessibility and bioactivity of compounds from algae. Trends Food Sci. Technol. 121, 114–128. https://doi.org/10.1016/j.tifs.2022.02.004.

Dias, J., Alvarez, M.J., Arzel, J., Corraze, G., Diez, A., Bautista, J.M., Kaushik, S.J., 2005. Dietary protein source affects lipid metabolism in the European seabass (*Dicentrarchus labrax*). Comp. Biochem. Physiol. Part A Mol. Integr. 142 (1), 19–31. https://doi.org/10.1016/j.cbpb.2005.07.005.

Din, N.A.S., Mohd Alayudin, A.S., Sofian-Seng, N.S., Rahman, H.A., Mohd Razali, N.S., Lim, S.J., Wan Mustapha, W.A., 2022. Algae as Functional Food Source of Fucoxanthin: a review. Foods 11, 2235. https://doi.org/10.3390/foods11152235.

Domozych, D.S., Ciancia, M., Fangel, J.U., Mikkelsen, M.D., Ulvskov, P., Willats, W.G., 2012. The cell walls of green algae: a journey through evolution and diversity. Front. Plant Sci. 3, 82. https://doi.org/10.3389/fpls.2012.00082.

Erlanger, B., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278. https://doi.org/10.1016/0003-9861(61)90145-X.

Felix, N., Brindo, R.A., 2014. Evaluation of raw and fermented seaweed, *Ulva lactuca* as feed ingredient in giant freshwater prawn *Macrobrachium rosenbergii*. Int. J. Fish. Aquat. Sci. 1 (3), 199–204.

Fernandes, T.J., Alves, R.C., Souza, T., Silva, J.M.G., Castro-Cunha, M., Valente, L.M., Oliveira, M.B.P.P., 2012. Lipid content and fatty acid profile of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles as affected by feed containing different amounts of plant protein sources. Food Chem. 134 (3), 1337–1342. https://doi.org/10.1016/j.foodchem.2012.03.026.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5.

Fonseca, F., Fuentes, J., Vizcaíno, A.J., Alarcón, F.J., Mancera, J.M., Martínez-Rodríguez, G., Martos-Sitcha, J.A., 2023. From invasion to fish fodder: Inclusion of the brown algae *Rugulopteryx okamurae* in aquafeeds for european sea bass *Dicentrarchus labrax* (L., 1758). Aquaculture 568, 739318. https://doi.org/10.1016/j.aquaculture.2023.739318.

Galafat, A., Vizcaíno, A.J., Sáez, M.I., Martínez, T.F., Jérez-Cepa, I., Mancera, J.M., Alarcón, F.J., 2020. Evaluation of *Arthrospira* sp. enzyme hydrolysate as dietary additive in gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Phycol. 32, 3089–3100. https://doi.org/10.1007/s10811-020-02141-0.

García-Gómez, J.C., Sempere-Valverde, J., González, A.R., Martínez-Chacón, M., Olaya-Ponzone, L., Sánchez-Moyano, E., Ostalé-Variberas, E., Megina, C., 2020. From exotic to invasive in record time: the extreme impact of *Rugulopteryx okamurae* (Dictyotales, Ochrophyta) in the strait of Gibraltar. Sci. Total Environ. 704, 135408 https://doi.org/10.1016/j.scitotenv.2019.135408.

García-Márquez, J., Vizcaíno, A.J., Barany, A., Galafat, A., Acién, G., Figueroa, F.L., Alarcón, F.J., Arijo, S., Abdala-Díaz, R.T., 2023. Evaluation of the combined administration of *Chlorella fusca* and *Vibrio proteolyticus* in diets for *Chelon labrosus*: effects on growth, metabolism, and digestive functionality. Animals 13 (4), 589. https://doi.org/10.3390/ani13040589.

Groisillier, A., Shao, Z., Michel, G., Goulitquer, S., Bonin, P., Krahulec, S., Nidetzky, B., Duan, D., Boyen, C., 2014. Mannitol metabolism in brown algae involves a new phosphatase family. J. Exp. Bot. 65 (2), 559–570. https://doi.org/10.1093/jxb/ert405.

Günerken, E., D'Hondt, E., Eppink, M.H.M., Garcia-Gonzalez, L., Elst, K., Wijffels, R., 2015. Cell disruption for microalgae biorefineries. Biotechnol. Adv. 33, 243–260. https://doi.org/10.1016/j.biotechadv.2015.01.008.

Haas, S., Bauer, J.L., Adakli, A., Meyer, S., Lippemeier, S., Schwarz, K., Schulz, C., 2016. Marine microalgae *Pavlova viridis* and *Nannochloropsis* sp. as n-3 PUFA source in diets for juvenile European sea bass (*Dicentrarchus labrax* L.). J. Appl. Phycol. 28, 1011–1021. https://doi.org/10.1007/s10811-015-0622-5.

Jerez-Cepa, I., Gorissen, M., Mancera, J.M., Ruiz-Jarabo, I., 2019. What can we learn from glucocorticoid administration in fish? Effects of cortisol and dexamethasone on intermediary metabolism of gilthead seabream (*Sparus aurata* L.). Comp. Biochem. Physiol. Part A Mol. Integr. 231, 1–10. https://doi.org/10.1016/j.cbpa.2019.01.010.

Keppler D., Decker K. 1974. Glycogen determination with amyloglucosidase. In Methods of Enzymatic Analysis (ed Bergmeyer, H. U.) 127–131 (New York, 1974).

Kumar, C.S., Ganesan, P., Suresh, P.V., Bhaskar, N., 2008. Seaweeds as a source of nutritionally beneficial compounds-a review. J. Food Sci. Technol. 45 (1), 1.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685. https://doi.org/10.1038/227680a0.

Li, X., Wang, X., Chen, D., Chen, S., 2011. Antioxidant activity and mechanism of protocatechuic acid in vitro. Funct. Foods Health Dis. 1 (7), 232–244. https://doi.org/10.31989/ffhd.v1i7.127.

Liu, M., Lia, W., Chenb, Y., Wana, X., Wanga, J., 2020. Fucoxanthin: a promising compound for human inflammation-related diseases. Life Sci. 255, 117850 https://doi.org/10.1016/j.lfs.2020.117850.

Messina, M., Piccolo, G., Tulli, F., Messina, C.M., Cardinaletti, G., Tibaldi, E., 2013. Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax* L.) fed diets containing wheat gluten and legume meals as substitutes for fish meal. Aquaculture 376, 6–14. https://doi.org/10.1016/j.aquaculture.2012.11.005.

Milligan, C.L., 2003. A regulatory role for cortisol in muscle glycogen metabolism in rainbow trout *Oncorhynchus mykiss* Walbaum. J. Exp. Biol. 206 (18), 3167–3173. https://doi.org/10.1242/jeb.00538.

Molina-Roque, L., Bárany, A., Sáez, M.I., Alarcón, F.J., Tapia, S.T., Fuentes, J., Mancera, J.M., Perera, E., Martos-Sitcha, J.A., 2022. Biotechnological treatment of microalgae enhances growth performance, hepatic carbohydrate metabolism and intestinal physiology in gilthead seabream (*Sparus aurata*) juveniles close to commercial size. Aquac. Rep. 25, 101248 https://doi.org/10.1016/j.aqrep.2022.101248.ç.

Moroney, N.C., Wan, A.H., Soler-Vila, A., FitzGerald, R.D., Johnson, M.P., Kerry, J.P., 2015. Inclusion of *Palmaria palmata* (red seaweed) in Atlantic salmon diets: effects on the quality, shelf-life parameters and sensory properties of fresh and cooked salmon fillets. J. Sci. Food Agric. 95 (5), 897–905. https://doi.org/10.1002/jsfa.6753.

Nesic, K., Habschied, K., Mastanjevic, K., 2021. Possibilities for the biological control of mycotoxins in food and feed. Toxins 13, 198. https://doi.org/10.3390/toxins13030198.

Peixoto, M.J., Ferraz, R., Magnon, L.J., Pereira, R., Gonçalves, J.F., Calduch-Giner, J., Pérez-Sánchez, J., Ozório, R.O., 2019. Protective effects of seaweed supplemented diet on antioxidant and immune responses in European seabass (*Dicentrarchus labrax*) subjected to bacterial infection. Sci. Rep. 9 (1), 16134 https://doi.org/10.1038/s41598-019-52693-6.

Peng, M., Xu, W., Mai, K.S., Zhou, H.H., Zhang, Y.J., Liufu, Z.G., Zhang, K., Aiet, Q., 2014. Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. Aquaculture 433, 442–449. https://doi.org/10.1016/j.aquaculture.2014.07.005.

Perera, E., Sanchez-Ruiz, D., Sáez, M.I., Galafat, A., Barany, A., Fernández-Castro, M., Vizcaíno, A.J., Fuentes, J., Martínez, T.F., Mancera, J.M., Alarcón, F.J., Martos-Sitcha, J.A., 2020. Low dietary inclusion of nutraceuticals from microalgae improves feed efficiency and modifies intermediary metabolisms in gilthead sea bream (*Sparus aurata*). Sci. Rep. 10, 1–14. https://doi.org/10.1038/s41598-020-75693-3.

Pérez-Jiménez, A., Peres, H., Rubio, V.C., Oliva-Teles, A., 2012. The effect of hypoxia on intermediary metabolism and oxidative status in gilthead sea bream (*Sparus aurata*) fed on diets supplemented with methionine and white tea. Comp. Biochem. Physiol. Part C. Toxicol. Pharmacol. 155, 506–516. https://doi.org/10.1016/j.cbpc.2011.12.005.

Pfleiderer, G., 1970. Particle-bound aminopeptidase from pig kidney. Methods Enzym. 19, 514–521. https://doi.org/10.1016/0076-6879(70)19038-0.

Remya, R.R., Samrot, A.V., Kumar, S.S., Mohanavel, V., Karthick, A., Chinnaiyan, V.K., Umapathy, D., Muhibbullah, M., 2022. Bioactive potential of brown algae. Adsorpt. Sci. Technol. 2022, 1–13. https://doi.org/10.1155/2022/9104835.

Rodríguez-Ruiz, J., Belarbi, E.H., Sanchez, J.L.G., Alonso, D.L., 1998. Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. Biotechnol. Tech. 12, 689–691. https://doi.org/10.1023/A:1008812904017.

Sáez, M.I., Vizcaíno, A., Galafat, A., Anguís, V., Fernández-Díaz, C., Balebona, M.C., Alarcón, F.J., Martínez, T.F., 2020. Assessment of long-term effects of the

macroalgae *Ulva ohnoi* included in diets on Senegalese sole (*Solea senegalensis*) fillet quality. Algal Res. 47, 101885 https://doi.org/10.1016/j.algal.2020.101885.

Sáez, M.I., Galafat, A., Vizcaíno, A.J., Rodríguez, C., Hernández de Rojas, A., Alarcón, F. J., Martínez, T.F., 2022. Long-term effect of a short pulse of dietary supplementation with algae on the quality of turbot (*Scophthalmus maximus*) frozen fillets. Front. Mar. Sci. 9, 957217 https://doi.org/10.3389/fmars.2022.957217.

Silva, F.C.P., Nicoli, J.R., Zambonino-Infante, J.L., Le Gall, M.M., Kaushik, S., Gatesoupe, F.J., 2010. Influence of partial substitution of dietary fish meal on the activity of digestive enzymes in the intestinal brush border membrane of gilthead sea bream, *Sparus aurata* and goldfish, *Carassius auratus*. Aquaculture 306, 233–237. https://doi.org/10.1016/j.aquaculture.2010.05.018.

Steen, F., Aragay, J., Zuljevic, A., Verbruggen, H., Mancuso, F.P., Bunker, F., Vitales, D., Gómez-Garreta, A., De Clerck, O., 2017. Tracing the introduction history of the brown seaweed *Dictyota cyanoloma* (*Phaeophyceae*, Dictyotales) in Europe. Eur. J. Phycol. 52 (1), 31–42. https://doi.org/10.1080/09670262.2016.1212998.

Sweetman, J., Dimitroglou, A., Davies, S.J., Torrecillas, S., 2008. Gut morphology: a kay to efficient nutrition. Int. Aquafeed 11, 26–30.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquacult. Res. 41 (5), 717–732. https://doi.org/10.1111/j.1365-2109.2008.02150.x.

Urán, P.A., Schrama, J.W., Rombout, J.H.W.M., Obach, A., Jensen, L., Koppe, W., Verreth, J.A.J., 2008. Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar* L.) at different temperatures. Aquac. Nutr. 14 (4), 324–330. https://doi.org/10.1111/j.1365-2095.2007.00534.x.

Van Der Boon, J., Van Den Thillart, G.E., Addink, A.D., 1991. The effects of cortisol administration on intermediary metabolism in teleost fish. Comp. Biochem. Physiol. Mol. 100 (1), 47–53. https://doi.org/10.1016/0300-9629(91)90182-C.

Vega, J., Catalá, T.S., García-Márquez, J., Speidel, L., Kunz, C., Geisler, C., Figueroa, F.L., 2023. Molecular diversity and biochemical content in two invasive alien species: looking for chemical similarities and bioactivities. Mar. Drugs 21, 5. https://doi.org/10.3390/md21010005.

Vizcaíno, A.J., López, G., Sáez, M.I., Jiménez, J.A., Barros, A., Hidalgo, L., Camacho-Rodríguez, J., Martínez, T.F., Cerón-García, M.C., Alarcón, F.J., 2014. Effects of the microalga *Scenedesmus almeriensis* as fishmeal alternative in diets for gilthead sea bream, *Sparus aurata*, juveniles. Aquaculture 431, 34–43. https://doi.org/10.1016/j.aquaculture.2014.05.010.

Vizcaíno, A.J., Mendes, S.I., Varela, J.L., Ruiz-Jarabo, I., Rico, R., Figueroa, F.L., Abdala, R., Moríñigo, M.A., Mancera, J.M., Alarcón, F.J., 2016. Growth, tissue metabolites and digestive functionality in *Sparus aurata* juveniles fed different levels of macroalgae, *Gracilaria cornea* and *Ulva rigida*. Aquac. Res. 47 (10), 3224–3238. https://doi.org/10.1111/are.12774.

Vizcaíno, A.J., Fumanal, M., Sáez, M.I., Martínez, T.F., Moriñigo, M.A., Fernández-Díaz, C., Anguís, V., Balebona, M.C., Alarcón, F.J., 2019. Evaluation of *Ulva ohnoi* as functional dietary ingredient in juvenile Senegalese sole (*Solea senegalensis*): effects on the structure and functionality of the intestinal mucosa. Algal Res. 42, 101608 https://doi.org/10.1016/j.algal.2019.101608.

Vizcaíno, A.J., Galafat, A., Sáez, M.I., Martínez, T.F., Alarcón, F.J., 2020. Partial characterization of protease inhibitors of *Ulva ohnoi* and their effect on digestive proteases of marine fish. Mar. Drugs 18 (6), 319. https://doi.org/10.3390/md18060319.

Wan, A.H.L., Davies, S.J., Soler-Vila, A., Fitzgerald, R., Johnson, M.P., 2019. Macroalgae as a sustainable aquafeed ingredient. Rev. Aquac. 11 (3), 458–492. https://doi.org/10.1111/raq.12241.

Zambonino-Infante, J.L., Gisbert, E., Sarasquete, S., Navarro, I., Gutiérrez, J., Cahu, C.L., 2008. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino, J.E.P., Bureau, D., Kapoor, B.G. (Eds.), Feeding and Digestive Functions in Fishes. Science Publishers, Enfield, NH, pp. 281–348.

Zhang, C., Rahimnejad, S., Wang, Y.R., Lu, K., Song, K., Wang, L., Mai, K., 2018. Substituting fish meal with soybean meal in diets for Japanese seabass (*Lateolabrax japonicus*): effects on growth, digestive enzymes activity, gut histology, and expression of gut inflammatory and transporter genes. Aquaculture 483, 173–182. https://doi.org/10.1016/j.aquaculture.2017.10.029.