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Use of technological processing of seaweed and microalgae as strategy to improve their apparent digestibility coefficients in European seabass

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78	Abstract	<p>Algae are natural sources of nutrients, but the presence of anti-nutritional factors often compromises nutrient apparent digestibility coefficients (ADCs) in several fish species. In this study, physical-mechanical and enzymatic technological processing was applied to two seaweeds (<i>Gracilaria gracilis</i> and <i>Ulva rigida</i>) and three microalgae (<i>Nannochloropsis oceanica</i>, <i>Chlorella vulgaris</i>, and <i>Tetraselmis</i> sp.) in order to evaluate its effectiveness in improving nutrient ADC values in diets for European seabass. A practical commercial-based diet was used as reference (REF) and experimental diets were prepared by replacing 30% of REF diet with each test alga used either intact or after processing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fast performance liquid chromatography (FPLC) analyses revealed that enzymatic processing was more effective than the physical one in changing the protein and peptides composition, increasing the amount of low-molecular-weight compounds in seaweeds and <i>N. oceanica</i> microalgae. Protein digestibility was significantly affected by algae species and in the case of the microalgae by the technological process. <i>Gracilaria gracilis</i> is better digested than <i>U. rigida</i> and physical processing enhanced protein and energy ADC values. <i>Nannochloropsis oceanica</i> and <i>C. vulgaris</i> are better digested than <i>Tetraselmis</i> sp.; the highest protein and energy ADCs were observed in diets containing enzymatically processed <i>N. oceanica</i> (NAN-ENZ) and physically processed <i>C. vulgaris</i> (CHLO-PHY), followed by the diet with physically processed <i>Tetraselmis</i> sp. (TETR-PHY). Results clearly showed that it is possible to increase nutrient accessibility and digestibility of algae by fish, by selecting the most adequate method to disrupt the cell wall. Moreover, the physical-mechanical and enzymatic technological processes used in this study are scalable to the industrial level.</p>	
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Use of technological processing of seaweed and microalgae as strategy to improve their apparent digestibility coefficients in European seabass (*Dicentrarchus labrax*) juveniles

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Abstract

Algae are natural sources of nutrients, but the presence of anti-nutritional factors often compromises nutrient apparent digestibility coefficients (ADCs) in several fish species. In this study, physical-mechanical and enzymatic technological processing was applied to two seaweeds (*Gracilaria gracilis* and *Ulva rigida*) and three microalgae (*Nannochloropsis oceanica*, *Chlorella vulgaris*, and *Tetraselmis* sp.) in order to evaluate its effectiveness in improving nutrient ADC values in diets for European seabass. A practical commercial-based diet was used as reference (REF) and experimental diets were prepared by replacing 30% of REF diet with each test alga used either intact or after processing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fast performance liquid chromatography (FPLC) analyses revealed that enzymatic processing was more effective than the physical one in changing the protein and peptides composition, increasing the amount of low-molecular-weight compounds in seaweeds and *N. oceanica* microalgae. Protein digestibility was significantly affected by algae species and in the case of the microalgae by the technological process. *Gracilaria gracilis* is better digested than *U. rigida* and physical processing enhanced protein and energy ADC values. *Nannochloropsis oceanica* and *C. vulgaris* are better digested than *Tetraselmis* sp.; the highest protein and energy ADCs were observed in diets containing enzymatically processed *N. oceanica* (NAN-ENZ) and physically processed *C. vulgaris* (CHLO-PHY), followed by the diet with physically processed *Tetraselmis* sp. (TETR-PHY). Results clearly showed that it is possible to increase nutrient accessibility and digestibility of algae by fish, by selecting the most adequate method to disrupt the cell wall. Moreover, the physical-mechanical and enzymatic technological processes used in this study are scalable to the industrial level.

Keywords Algae · Antinutritional factors (ANFS) · Aquafeeds · Cell wall-rupture · Nutrient digestibility (ADC) · Novel ingredients

Introduction

The sustainable growth of aquaculture largely depends on the use of novel nutrient sources to replace fish meal (FM) and

fish oil (FO), without compromising fish growth and welfare, and still assuring the nutritional value of end products (Naylor et al. 2009). Plants have been largely used to partially replace FM and FO, but lack omega-3 LC-PUFA (Turchini et al.

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2010) and directly compete with animal and human nutrition (Gatlin et al. 2007). Algae-based ingredients have recently attracted the attention of the feed industry sector as sustainable sources of nutrients (Becker 2007; Wan et al. 2019) and they also contain many bioactive compounds like pigments, vitamins, and minerals with a large spectrum of biological activities (Holdt and Kraan 2011; Bellou et al. 2014; Araújo et al. 2016; Valente et al. 2016; Neto et al. 2018; Pereira et al. 2019; Batista et al. 2020). However, the ability of higher trophic level carnivorous fish, like European seabass *Dicentrarchus labrax*, to effectively extract nutrients from algal species is hampered by the high complexity of their cell walls, which may introduce anti-nutritional factors (Neto et al. 2018; Tibbetts 2018; Zheng et al. 2020); these may harm the intestinal tract and result in inflammation and reduced nutrient uptake (Araújo et al. 2016; Moutinho et al. 2018; Granby et al. 2020). Previous studies reported morphological alterations in the intestine of several fish species fed algal biomass, namely reduction of absorption area and epithelial degeneration (Atalah et al. 2007; Silva et al. 2015; Araújo et al. 2016; Moutinho et al. 2018). Moreover, several microalgae have highly recalcitrant cell walls and high carbohydrate content that negatively affect the activity of digestive enzymes (Skrede et al. 2011; Tibbetts 2018). Likewise, the presence of indigestible fibers in seaweeds (e.g., lectins), resistant to digestive enzymes, may affect their nutrient bioavailability (Wells et al. 2017; Zheng et al. 2020). The type of algal carbohydrates can affect the activity of digestive enzymes, in particular those located in the brush border membrane of the enterocyte, which is responsible for the final stages of degradation and assimilation of food (Perez-Jimenez et al. 2015).

To establish algal biomass as a sustainable next-generation ingredient, economically feasible processing technologies, able to disrupt cell walls, concentrate nutrients, and enhance nutrient bioavailability for fish, need to be developed (Tibbetts 2018). Application of such processing techniques would release proteins, lipids, and other naturally hydrophobic components and increase their digestion and nutrient absorption rate by fish (Tulli et al. 2017). Cell wall disruption and cell disintegration can be achieved through mechanical technologies (bead-beating, milling, ultrasonication, high-pressure homogenization, and spray-drying), thermal (microwave, autoclaving, and freezing), chemical (organic solvents, osmotic shock, and acid-alkali reactions), or biological processes (microbial degradation and enzymatic reactions) (Lee et al. 2012; Ometto et al. 2014; Günerken et al. 2015; Agboola et al. 2019). However, some of these disruption methods (e.g., bead milling, microwave, and ultrasonication) have high energy consumption, restricting their industrial applications (Günerken et al. 2015). Currently, enzymatic cell disruption has delivered effective and cost-competitive results when compared to mechanical and chemical cell

disruption methods (Demuez et al. 2015). The use of enzymes (e.g., lipase, pectinase, cellulase, and protease), single or mixed, and chemical procedures are considered of great interest as they can break down the polysaccharide and complex proteins allowing the release of smaller molecules such as peptides, some of them with bioactive properties, providing additional physiological health benefits to fish. The specificity of the selected enzymes plays an important role in the efficiency of cell degradation, leading to the frequent application of enzyme mixtures to target different structural molecules. However, the introduction of enzymes implies additional associated cost within the overall process (Demuez et al. 2015). For this reason, low-cost enzymatic mixtures used in combination with mechanical disruption may maximize the release of soluble protein and peptides in the algae biomass coupled with reduced processing price.

This work aims to evaluate the effectiveness of cost-effective physical-mechanical and enzymatic technological processes, applied to two seaweeds (*Gracilaria gracilis* and *Ulva rigida*) and three microalgae (*Nannochloropsis oceanica*, *Chlorella vulgaris*, and *Tetraselmis* sp.), in improving nutrient apparent digestibility coefficients (ADCs) in diets for European seabass juveniles.

Materials and methods

The present study was directed and performed by accredited scientists in laboratory animal science by the national competent authority (Direção Geral de Alimentação e Veterinária, DGAV) at a facility with permission to conduct experiments on fish, in compliance with the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto-Lei no. 113/2013, de 7 de Agosto) on the protection of animals used for scientific purposes. All animal procedures were subject to an ethical review process carried out by CIIMAR animal welfare body (ORBEA-CIIMAR) and further approved by DGAV.

Ingredients

Two commercial IMTA-cultivated seaweed (*U. rigida* and *G. gracilis*) produced by ALGApplus (Ílhavo, Portugal) and three microalgae (*N. oceanica*, *C. vulgaris*, and *Tetraselmis* sp.) produced under industrial scale by Allmicroalgae (Pataias, Portugal) were dried by convection and by spray-dryer, respectively, before being used in this experiment. The selected algae were either used entirely (not processed, NO), or previously submitted to technological processing (Valente et al. 2019b) before being included in the test diets. The proximate composition, amino acid profile, and mineral content of each alga biomass are presented in Table 1. When

t1.1 **Table 1** Proximate composition,
t1.2 amino acid profile and mineral
content of test algae without
t1.3 processing (% or kJ g⁻¹ dry matter
basis)

	<i>Ulva rigida</i>	<i>Gracilaria gracilis</i>	<i>Nannochloropsis oceanica</i>	<i>Chlorella vulgaris</i>	<i>Tetraselmis</i> sp.	
t1.4	Dry matter (% DM)	83.3	89.7	93.8	96.1	95.2
t1.5	Crude protein	15.1	34.5	34.7	54.0	26.3
t1.6	Crude fat	1.2	0.6	10.2	9.9	1.4
t1.7	Ash	30.1	19.4	36.1	12.7	34.1
t1.8	Carbohydrates ¹	36.9	35.2	12.8	19.5	33.4
t1.9	Gross energy	11.5	15.4	16.2	20.9	13.3
t1.10	Neutral detergent fiber	26.4	26.7	30.3	37.3	11.5
t1.11	EAA (% DM)	7.2	13.9	20.1	27.3	12.6
t1.12	Arginine	0.5	1.3	2.0	2.7	1.3
t1.13	Histidine	0.1	0.2	0.6	0.4	0.3
t1.14	Lysine	0.9	1.6	3.2	3.6	1.6
t1.15	Threonine	0.8	1.7	1.9	3.3	1.2
t1.16	Isoleucine	1.0	2.3	2.4	3.8	1.6
t1.17	Leucine	1.1	1.9	3.1	4.1	2.1
t1.18	Valine	1.7	3.1	5.1	6.1	2.8
t1.19	Methionine	0.2	0.2	vest	0.6	0.3
t1.20	Phenylalanine	0.8	1.7	1.8	2.7	1.4
t1.21	CEAA (% DM)	0.8	1.4	1.7	2.3	1.1
t1.22	Cystine	vest	0.4	vest	0.3	0.1
t1.23	Hydroxyproline	0.2	ND	vest	vest	vest
t1.24	Proline	0.6	1.0	1.7	2.0	1.0
t1.25	NEAA (% DM)	7.0	10.9	12.9	22.2	7.6
t1.26	Alanine	1.5	1.9	2.1	4.5	1.6
t1.27	Tyrosine	0.6	1.3	1.1	2.7	0.7
t1.28	Aspartate	1.4	2.6	2.9	4.5	1.5
t1.29	Glutamate	1.9	2.4	3.9	5.7	1.6
t1.30	Glycine	0.7	1.1	1.7	2.2	1.2
t1.31	Serine	0.9	1.6	1.3	2.7	0.9
t1.32	Minerals (mg g ⁻¹)					
t1.33	B	0.03	0.08	0.05	vest	0.03
t1.34	Ca	4.12	1.11	2.27	5.54	10.8
t1.35	Cu	0.02	0.01	0.01	0.07	0.01
t1.36	Fe	0.30	0.57	0.39	0.98	0.29
t1.37	K	21.02	53.29	21.20	21.61	28.74
t1.38	Mg	30.36	1.80	17.37	2.82	18.13
t1.39	Mn	0.05	0.15	0.04	0.06	0.03
t1.40	Na	24.07	8.51	55.97	7.07	44.02
t1.41	P	1.58	4.64	7.27	21.12	5.61
t1.42	Zn	0.01	0.02	0.07	0.19	0.03

EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; vest, vestigial amount of amino acid (<0.01 mg g⁻¹)

¹ Calculated as 100 – (ash + crude protein + crude fat + moisture)

140 processed, the resulting product was entirely used as test in-
141 gredient. The peptide size distribution of aqueous extracts of
142 the two seaweeds and three microalgae before and after pro-
143 cessing is presented in Table 2.

Technological processing of algae

144

Two technological processes were applied to unprocessed al-
gae biomass: a physical-mechanical rupture method (PHY) to

145

146

t2.1 **Table 2** Peptide size distribution and respective chromatogram area of aqueous extracts of the two seaweeds and three microalgae before and after processing

t2.2	Algae	Processing	Chromatogram area according to size distribution				Total area
			> 12.3 kDa	12.3–6.5 kDa	6.5–0.19 kDa	< 0.19 kDa	
t2.4	<i>Gracilaria gracilis</i>	NO	6837	1814	14,183	47,784	70,618
t2.5		PHY	13,692	4676	19,002	58,561	95,930
t2.6		ENZ	15,935	9325	72,084	77,971	175,315
t2.7	<i>Ulva rigida</i>	NO	6297	2950	5497	6095	20,839
t2.8		PHY	4084	4570	7581	9392	25,628
t2.9		ENZ	5200	9816	25,489	17,056	57,562
t2.10	<i>Nannochloropsis oceanica</i>	NO	5401	2167	16,809	10,815	35,193
t2.11		PHY	5984	1575	16,157	10,541	34,257
t2.12		ENZ	22,080	5134	44,766	17,383	89,364
t2.13	<i>Chlorella vulgaris</i>	NO	28,782	7120	34,655	54,550	125,106
t2.14		PHY	26,039	8079	47,256	84,715	166,089
t2.15		ENZ	20,944	7107	66,295	81,775	176,120
t2.16	<i>Tetraselmis</i> sp.	NO	11,202	2637	8788	9344	31,971
t2.17		PHY	12,340	2773	8184	8486	31,783
t2.18		ENZ	13,089	4863	22,962	15,433	56,348

NO, not processed; PHY, physically processed; ENZ, enzymatically processed

147 efficiently disrupt cell walls using a vibratory grinding mill
 148 and enzymatic lysis using a cocktail of enzymes applied to the
 149 physically disrupted algae (ENZ). The PHY process relied on
 150 the use of a vibratory mill (Siebtechnik TS250, Geldern,
 151 Germany) with a solid dense puck and one ring, for 1–
 152 5 min, generating a disrupted algal suspension. In the ENZ
 153 process, physically disrupted algal biomass was hydrolyzed
 154 with a commercial low-cost enzymatic cocktail (containing
 155 lipase, pectinase, cellulase, and amylase, New Enzymes,
 156 Lda., Maia, Portugal) at a pH 6–7, for 3 h (Valente et al.
 157 2019b). The yield in terms of recovered algae biomass was
 158 79% and 99%, for the PHY and ENZ process, respectively.
 159 The recovered biomass was then dried using industrial
 160 methods already employed for each algal biomass: seaweeds
 161 were dehydrated in a pilot-scale tray dryer (Armfield UOP8,
 162 Ringwood, England), with an airflow of 0.6 m s⁻¹ maintained
 163 at 50 °C, until constant weight of the sample was achieved;
 164 microalgae were dried in a pilot-scale spray dryer (Niro
 165 Atomizer 2394, Copenhagen, Denmark) with a vanned wheel
 166 rotating at high speed and a concurrent drying chamber (0.8 m
 167 diameter and 0.6 m height). The dried algae biomass was
 168 collected in a single cyclone air separator system.

169 Experimental diets

170 Based on the known nutritional requirements of European
 171 seabass, a commercial-based diet was formulated and extruded
 172 by SPAROS Lda. (Olhão, Portugal) and used as basal

173 mixture (Table 3). To this mixture, 10 g kg⁻¹ chromic oxide
 174 (Cr₂O₃, Merck KGaA, Germany) was added as an inert mark-
 175 er for the evaluation of the apparent digestibility coefficient
 176 (ADC) of nutrients and energy. The reference diet (REF)
 177 consisted of 1000 g kg⁻¹ of the basal mixture (Table 4).
 178 Fifteen test diets were prepared by mixing 700 g kg⁻¹ of the
 179 basal mixture and 300 g kg⁻¹ of each test ingredient: *U. rigida*
 180 (DULV), *G. gracilis* (DGRA), *N. oceanica* (DNAN),
 181 *C. vulgaris* (DCHLO), and *Tetraselmis* sp. (DTRET); each
 182 test ingredient was either used unprocessed (NO) or after
 183 PHY or ENZ processes. The dried algal biomass (either not
 184 processed or processed) was ground (< 250 μm) in a
 185 micropulverizer hammer mill (model SH1, Hosokawa-
 186 Alpine, Germany) prior addition to the basal mixture. Diets
 187 were manufactured with a pilot-scale twin-screw extruder
 188 (CLEXTRAL BC45, France) to a pellet size of 3 mm and
 189 oil was added after the extrusion process. All batches of ex-
 190 truded feeds were dried in a convection oven (OP 750-UF,
 191 LTE Scientifics, UK) and stored at 4 °C until use. The formu-
 192 lation and proximate composition of the experimental diets are
 193 shown in Tables 4 and 5.

Digestibility trial

194
 195 The digestibility trial was conducted at the Experimental
 196 Research Station of CCMAR (37° 00' N, 07° 58' W, Faro,
 197 Portugal) between November and December, with juvenile
 198 European seabass (*Dicentrarchus labrax*) obtained from

t3.1 **Table 3** Ingredient composition of the basal mixture

t3.2	Ingredients (%)	
t3.3	Fishmeal 70 ¹	5.0
t3.4	Fishmeal 60 ²	20.0
t3.5	Soy protein concentrate ³	12.0
t3.6	Pea protein concentrate ⁴	2.3
t3.7	Wheat gluten ⁵	5.5
t3.8	Corn gluten ⁶	8.0
t3.9	Soybean meal ⁷	15.0
t3.10	Rapeseed meal ⁸	5.0
t3.11	Wheat meal ⁹	11.3
t3.12	Fish oil ¹⁰	13.7
t3.13	Vit and min premix ¹¹	1.0
t3.14	Binder ¹²	0.2
t3.15	Chromic oxide ¹³	1.0
t3.16	Dry matter (DM, %)	95.2
t3.17	Crude protein (% DM)	48.7
t3.18	Crude fat (% DM)	13.5
t3.19	Carbohydrates (% DM) ¹⁴	22.8
t3.20	Gross energy (kJ g ⁻¹ DM)	21.9
t3.21	Ash (% DM)	10.2

¹ Peruvian fishmeal LT: 71.0% crude protein (CP), 11.0% crude fat (CF), EXALMAR, Peru;

² Fishmeal 60: 60% CP, 12% CF, Savinor SA, Portugal;

³ Soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands;

⁴ Pea protein concentrate: Nutralys F85F, 78% CP, 1% CF, Roquette, France;

⁵ Wheat gluten: 84% CP, 1.3% CF, Roquette, France;

⁶ Corn gluten meal: 61.0% CP, 6.0% CF, COPAM, Portugal;

⁷ Soybean meal 48: Dehulled solvent extracted soybean meal: 47.7% CP, 2.2% CF, Cargill, Spain;

⁸ Rapeseed meal: 36% CP, 2.7% CF, PREMIX Lda, Portugal;

⁹ Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal;

¹⁰ Savinor S.A., Portugal;

¹¹ Vitamin and mineral premix: INVIVO 1%, Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg kg⁻¹ diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg kg⁻¹ diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings;

¹² Kielseguhr (natural zeolite): LIGRANA GmbH, Germany;

¹³ Cr₂O₃; Merck KGaA, Germany;

¹⁴ Calculated by estimation: 100 - (ash + crude protein + crude fat + moisture)

chromic oxide) and adapted over 4 weeks to the experimental conditions in quarantine. Subsequently, thirteen homogeneous groups of twelve fish (bodyweight 62 ± 8.6 g) were randomly distributed by thirteen tanks of 50 L with individual feces sedimentation columns (Guelph system), designed according to Cho and Slinger (1979) supplied with flow-through seawater. Fish were then adapted to the experimental conditions for 15 days (water temperature of 21 ± 1.8 °C, salinity of 35 g L⁻¹, flow rate at 3 L min⁻¹, and natural photoperiod corresponding to 10–11 h daylight length). After the adaptation period, fish were fed the experimental diets in a daily meal until visual satiation for 5 days a week during the feces collection period. Diets were tested in triplicate. All diets were accepted by the fish and no mortality was observed during the digestibility trial. About 30 min after feeding, every tank was carefully cleaned to assure that no uneaten pellet was left in the tanks and the sedimentation column. Feces were collected from the sedimentation column every morning, before feeding, and then centrifuged (7200 rpm for 5 min) to eliminate water excess before freezing at -20 °C. Daily collection of the feces was performed for each experimental diet following previous seabass digestibility studies (Campos et al. 2018; Monteiro et al. 2018) until collecting the necessary amount of feces to perform all required analysis (8–17 days). Since the rearing system used consisted of thirteen tanks, this procedure was repeated over time until all ingredients were tested in triplicate. Each replicate was carried out in a different group of fish (tank) to reduce any tank effect. Fish were fasted for 24 h between the collecting period of different diets, allowing the first 5 days of feeding for adaption to the new diet. The remaining procedure was performed as described above. At the end of the trial, all feces were freeze-dried prior to analysis.

The apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Maynard et al. (1979): $ADC (\%) = 100 \times (1 - (\text{dietary } Cr_2O_3 \text{ level} / \text{feces } Cr_2O_3 \text{ level}) \times (\text{feces nutrient or energy level} / \text{dietary nutrient or energy level}))$. ADC of dry matter was calculated as follows: $ADC (\%) = 100 \times (1 - (\text{dietary } Cr_2O_3 \text{ level} / \text{feces } Cr_2O_3 \text{ level}))$. The ADCs of nutrients and energy of the test ingredients were estimated according to NRC (2011): $ADC_{ing} (\%) = ADC_{test} + [(ADC_{test} - ADC_{ref}) \times ((0.7 \times D_{ref}) / (0.3 \times D_{ing}))]$; where ADC_{test} = ADC (%) of the experimental diet, ADC_{ref} = ADC (%) of the reference diet, D_{ref} = g kg⁻¹ nutrient (or kJ kg⁻¹ gross energy) of the reference diet (DM basis); D_{ing} = g kg⁻¹ nutrient (or kJ kg⁻¹ gross energy) of the test ingredient (DM basis). The digestible amino acids (DAAs) content of each algae meal was calculated as follows: $DAA (mg g^{-1} DM) = ADC \text{ of the amino acid in the test ingredient} \times AA_{ing}$, where AA_{ing} = mg g⁻¹ amino acid of the test ingredient (DM basis).

199 Acuicultura Y Nutricion De Galicia S.L. (Ortoño, Spain).
200 Upon arrival, fish were fed the reference diet (without

Q3 t4.1 **Table 4** Formulation and proximate composition of the experimental diets

		Experimental diets															
		REF	DULV			DGRA			DNAN			DCHLO			DTETR		
			NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ
t4.5	Basal mix (g kg ⁻¹)	1000	700	700	700	700	700	700	700	700	700	700	700	700	700	700	700
t4.6	<i>Ulva rigida</i>		300														
t4.7	<i>U. rigida</i> physically processed			300													
t4.8	<i>U. rigida</i> enzymatically processed				300												
t4.9	<i>Gracilaria gracilis</i>					300											
t4.10	<i>G. gracilis</i> physically processed						300										
t4.11	<i>G. gracilis</i> enzymatically processed							300									
t4.12	<i>Nannochloropsis oceanica</i>								300								
t4.13	<i>N. oceanica</i> physically processed									300							
t4.14	<i>N. oceanica</i> enzymatically processed										300						
t4.15	<i>Chlorella vulgaris</i>											300					
t4.16	<i>C. vulgaris</i> physically processed												300				
t4.17	<i>C. vulgaris</i> enzymatically processed													300			
t4.18	<i>Tetraselmis</i> sp.														300		
t4.19	<i>Tetraselmis</i> sp. physically processed															300	
t4.20	<i>Tetraselmis</i> sp. enzymatically processed																300
t4.21	Proximate composition (% or kJ g ⁻¹ DM)																
t4.22	Dry matter (DM, %)	95.2	89.8	92.3	92.3	91.7	92.8	91.6	90.0	93.8	93.0	92.2	93.8	94.1	90.9	92.9	92.5
t4.23	Crude protein	48.7	38.6	39.0	38.6	44.1	44.1	42.9	44.6	43.8	44.2	51.2	50.1	48.9	41.7	41.4	41.2
t4.24	Crude fat	13.5	11.9	10.3	9.3	11.0	10.5	11.6	12.3	11.9	11.7	11.6	10.8	12.4	11.0	9.7	10.8
t4.25	Carbohydrates ¹	22.8	23.8	27.4	26.8	23.3	25	23	14.3	19.9	19.3	18.3	21.8	21.9	20.2	24.2	23.3
t4.26	Gross energy	21.9	19.0	20.4	21.1	20.3	23.2	22.5	22.0	21.0	22.9	22.5	23.6	24.8	20.3	21.2	22.5
t4.27	Ash	10.2	15.5	15.6	17.6	13.3	13.2	14.1	18.8	18.2	17.8	11.1	11.1	10.9	18.0	17.6	17.2

REF, reference diet; DULV, diet with 30% *U. rigida*; DGRA, diet with 30% *G. gracilis*; DNAN, diet with 30% *N. oceanica*; DCHLO, diet with 30% *C. vulgaris*; DTETR, diet with 30% *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. ¹ Calculated by estimation: 100 - (ash + crude protein + crude fat + moisture)

254 **Chemical analysis**

255 Each test ingredient, experimental diet, and feces were ground
 256 (feces were sifted) and homogenized before analysis.
 257 Proximate composition analysis was performed in duplicate.
 258 All samples were analyzed for dry matter (105 °C for 24 h),
 259 ash by combustion in a muffle furnace (Nabertherm L9/11/
 260 B170, Germany; 500 °C for 5 h), crude protein (N × 6.25)
 261 using a Leco nitrogen analyzer (Model FP-528, Leco
 262 Corporation, USA), total lipid content according to Folch
 263 et al. (1957), and gross energy by an adiabatic bomb calorim-
 264 eter (Werke C2000, IKA, Germany). Chromic oxide content
 265 in diets and feces was determined according to Bolin et al.
 266 (1952).

267 Algae crude fiber content was analyzed as neutral detergent
 268 fiber (NDF) according to ISO 16472:2006 (Robertson and
 269 Van Soest 1981; Van Soest and Robertson 1985); carbohy-
 270 drates of the test ingredients were calculated by deducting the

sum of ash, CP, and total lipids from DM. The mineral content 271
 of the algae was determined according to USEPA (1995). 272
 Aliquots (0.3 g) of dry microalgae biomass were introduced 273
 in Teflon® microwave vessels and 9 mL of concentrated 274
 HNO₃ + 1.0 mL aqua regia was added. Samples were proc- 275
 essed in a microwave digester (CEM Mars Xpress Matthews, 276
 USA) at 175 °C and elevated frequency of 2450 MHz. The 277
 temperature was kept at 170–180 °C for 10 min. After 278
 cooling, digested solutions were filtered through a PTFE filter 279
 (0.2 µm size), transferred into 20-mL volumetric flasks and 280
 stored at 5 °C for determination by Inductively Coupled 281
 Plasma Optical Emission Spectroscopy (ICP-OES). A 282
 Varian Vista Pro axial instrument (Varian Inc., USA) 283
 equipped with a cross-flow nebulizer and auto-sampler was 284
 used. The calibration was performed using an ICP-standard 285
 elements solution in 5% HNO₃ (Merck solution IV) using 286
 yttrium (Y) as an internal standard. The calibration curve 287
 and two blanks were run during each set of analyses, to check 288

t5.1 **Table 5** Amino acid profile of the experimental diets (% dry matter basis)

		Experimental diets															
		REF	DULV			DGRA			DNAN			DCHLO			DTETR		
			NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ
t5.5	EAA (% DM)	25.5	20.3	27.0	22.9	22.1	19.4	21.0	24.4	27.0	21.9	26.6	27.1	24.5	23.8	25.1	26.5
t5.6	Arginine	2.9	2.6	3.4	2.9	3.0	2.5	2.2	2.3	3.2	2.4	3.4	3.1	2.9	3.0	2.8	3.4
t5.7	Histidine	1.0	0.7	0.9	0.9	0.8	0.7	0.9	1.0	1.1	0.9	1.0	1.2	0.7	0.7	1.0	1.1
t5.8	Lysine	3.9	2.3	3.8	3.2	2.7	2.2	2.4	2.5	3.0	2.5	3.4	2.7	3.0	3.6	2.7	3.1
t5.9	Threonine	2.2	2.0	2.6	2.3	2.2	1.7	2.0	2.3	2.6	2.1	2.6	2.5	2.2	2.2	2.3	2.7
t5.10	Isoleucine	3.6	2.3	3.6	2.4	2.6	2.5	2.7	3.3	3.9	3.1	2.9	3.4	3.2	3.3	3.5	3.3
t5.11	Leucine	3.9	3.4	4.3	3.7	3.5	3.1	3.6	4.1	4.4	3.6	4.4	4.6	3.9	3.6	4.0	3.9
t5.12	Valine	4.5	3.9	5.0	4.2	4.3	4.0	4.0	5.4	5.3	4.2	5.1	5.9	5.3	4.3	5.7	5.3
t5.13	Methionine	1.1	1.2	0.9	1.2	1.1	0.9	1.2	1.3	1.1	0.9	1.4	1.2	1.1	1.0	1.1	1.3
t5.14	Phenylalanine	2.3	1.8	2.3	2.0	1.8	1.7	1.9	2.2	2.4	2.1	2.4	2.5	2.1	2.1	2.0	2.4
t5.15	CEAA (% DM)	3.8	2.9	3.5	3.5	3.0	2.8	3.1	3.6	3.8	3.3	3.6	3.8	3.4	3.2	3.5	3.5
t5.16	Cystine	0.5	0.3	vest	0.4	0.4	0.3	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
t5.17	Hydroxyproline	0.6	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.5	0.3	0.3
t5.18	Proline	2.7	2.3	3.2	2.7	2.4	2.2	2.5	3.0	3.1	2.6	3.0	3.1	2.7	2.3	2.9	2.9
t5.19	NEAA (% DM)	20.0	17.9	22.7	20.3	20.1	18.0	19.9	20.3	23.0	18.9	22.0	25.2	20.0	21.7	23.0	23.5
t5.20	Alanine	2.0	1.8	3.0	2.1	2.0	1.9	2.2	2.5	2.6	2.2	2.6	2.8	2.3	1.9	2.7	2.9
t5.21	Tyrosine	1.7	1.2	1.5	1.5	1.3	1.1	1.3	1.5	1.6	1.4	1.7	1.8	1.5	1.7	1.4	1.7
t5.22	Aspartate	4.2	3.2	4.3	3.6	4.1	3.5	3.6	4.3	4.7	3.8	4.7	4.8	4.0	4.4	4.7	4.2
t5.23	Glutamate	7.4	7.7	8.6	8.8	8.6	8.0	9.0	7.4	9.2	7.1	8.1	10.8	8.0	9.7	9.4	10.0
t5.24	Glycine	2.2	1.7	2.6	2.3	1.9	1.7	1.9	2.3	2.4	2.2	2.3	2.6	2.1	1.8	2.4	2.4
t5.25	Serine	2.5	2.2	2.7	2.1	2.2	1.8	2.0	2.2	2.4	2.2	2.5	2.5	2.0	2.3	2.5	2.3

REF, reference diet; DULV, diet with 30% *U. rigida*; DGRA, diet with 30% *G. gracilis*; DNAN, diet with 30% *N. oceanica*; DCHLO, diet with 30% *C. vulgaris*; DTETR, diet with 30% *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids. vest, vestigial amount of amino acid (<0.1%)

289 the purity of the chemicals. The method detection limit
290 (MDL) was calculated as $3 s/m$ (where s is the standard deviation
291 of 10 replicate blanks and m is the slope of the calibration
292 curve) for each element.

293 To measure the amino acid profile of test ingredients, ex-
294 perimental diets and feces samples were subjected to acid
295 hydrolysis (6 M HCl) in an oven for 18 h at 110 °C. The
296 hydrolysis was performed using an amount of the samples
297 corresponding to 5–10 mg protein per mL HCl. After hydro-
298 lysis, the samples were cooled to room temperature (RT °C)
299 and 100 µL was diluted with 1.5 mL 1 M NaCO₃ and filtered
300 through a 0.2-µm syringe filter (Q-max PTFE, Ø13mm,
301 Frisette ApS, Denmark) before derivatization using the
302 EZ:Faast™ Amino Acid Analysis kit from Phenomenex
303 (SA). The samples (50 µL) were then analyzed by
304 LC-(APCI)-MS (Agilent 1100, Agilent Technology) accord-
305 ing to the procedure described by Sabeena Farvin et al. (2010).

306 Protein pattern types of all algal extracts were evaluated by
307 sodium dodecyl sulfate-polyacrylamide gel electrophoresis
308 (SDS-PAGE) in Mighty Small (Hoefer) slab cell according

309 to the method of (Laemmli 1970), using 12% acrylamide 309
310 ($C = 2.6\%$, w/w) slab gels (1.5 mm thick). The algae extracts 310
311 were obtained by adding 2 mL 1% sodium dodecyl sulfate 311
312 (SDS), 100 mM dithiothreitol (DTT) and 60 mM Tris HCl 312
313 (pH 8.3) to 50 mg of each of the dried algae. After gentle 313
314 shaking at room temperature for 1 h, samples were homoge- 314
315 nized (Polytron PT 1200, Kinematica) for 30 s, boiled for 315
316 2 min, and incubated at room temperature for 30 min. The 316
317 samples were then homogenized and boiled again for 2 min 317
318 and centrifuged for 15 min at 20 °C at 20000×g. The super- 318
319 natant was collected as sample extract. Extract aliquots were 319
320 diluted 1:1 with sample buffer containing 125 mM Tris HCl 320
321 (pH 6.8), 2.4% SDS, 50 mM DTT, 10% v/v glycerol, 0.5 mM 321
322 EDTA and bromophenol blue. Each lane was loaded with 322
323 20 µL sample, corresponding to 0.25 mg algae. Mark12 323
324 (Novex, USA) was used as molecular weight markers. The 324
325 electrophoresis was run at 100 V for 15 min followed by 325
326 150 V for 1 h (max. 40 mA per gel) and afterwards the gels 326
327 were stained using colloidal Coomassie Brilliant Blue, accord- 327
328 ing to Rabilloud and Charmont (2000). To further evaluate the 328

329 effects of processing techniques on the algae, size exclusion
 330 chromatography by fast performance liquid chromatography
 331 (FPLC) was performed on algae aqueous extracts to charac-
 332 terize smaller proteins/peptides of molecular weights below
 333 approximately 20–30 kDa. The processed algae (100 mg)
 334 was extracted in 2 mL of water by homogenization
 335 (Polytron PT 1200, Kinematica) for 30 s, incubation at RT
 336 °C for 30 min followed by a new homogenization (30 s),
 337 and an incubation for 15 min (RT °C). The sample was then
 338 centrifuged for 15 min at 20 °C at 20000×g and the supernat-
 339 ant was filtered (0.2 µm) before analysis on fast performance
 340 liquid chromatography (FPLC) equipment (Äkta Purifier sys-
 341 tem with Frac 950 collector, GE Healthcare Life Sciences,
 342 UK). The sample (100 µL; corresponding to 5 mg algae)
 343 was injected onto a Superdex™ peptide 10/300 GL column
 344 (GE Healthcare), using a 100 mM ammonium acetate, pH 8 as
 345 running buffer at a flow rate of 0.25 mL min⁻¹. Eluting com-
 346 pounds were detected at 215 nm. Cytochrome C (CytC,
 347 12.3 kDa), aprotinin (6.5 kDa), and triglycine Gly3 (189 Da)
 348 were used as external molecular weights standards.

349 Statistical analysis

350 Seaweed and microalgae results are presented separately.
 351 ADCs data were tested for normality and homogeneity of
 352 variances by Shapiro-Wilk and Levene's tests, respectively,
 353 and transformed whenever required before being submitted to
 354 a one-way ANOVA (for diets ADC) and two-way ANOVA
 355 (for ingredients ADC), with the statistical program IBM SPSS
 356 STATISTICS, 25.0 package, IBM Corporation, USA). When
 357 appropriate, individual means were compared using HSD

Tukey Test. When data did not meet the assumptions of
 ANOVA, a non-parametric test, Kruskal Wallis test was per-
 formed and the pairwise multiple comparison of mean ranks,
 were carried out to identify significant differences between
 groups. In all cases, the minimum level of significance was
 set at $p < 0.05$.

Results

The proximate composition of the test algae in their no-
 processed form (NO) varied enormously among species
 (Table 1). In terms of dry matter basis, crude protein content
 ranged from 15.1 (*U. rigida*) to 54.0% (*C. vulgaris*), crude fat
 varied between 0.6 (*G. gracilis*) and 10.2% (*N. oceanica*), and
 gross energy from 11.5 (*U. rigida*) to 20.9 kJ g⁻¹ (*C. vulgaris*).
 Neutral detergent fiber varied from 11.5 in *Tetraselmis* sp. to
 37.3% in *C. vulgaris*. Ash content varied between 13 and
 36%, being lowest in *C. vulgaris* followed by *G. gracilis*.
 The amino acid profile and mineral content also showed great
 variation among algae. *C. vulgaris* had the highest EAA con-
 tent (27.3% DM), followed by *N. oceanica* (20.1%) and both
 were particularly rich in lysine (3.2–3.6) and valine (5.1–6.1).
U. rigida had the lowest EAA content (7.2%), but is a rich
 source of Na, K, and Mg. Among selected algae, *G. gracilis* is
 the richest source of K and *C. vulgaris* is rich in P.

SDS PAGE was used to characterize the processing effects
 on the protein composition in the alga ingredients selected for
 this study by comparing the different algae to their processed
 counterparts (Fig. 1). The tested no-processed (NO) algae had
 different protein profiles (Fig. 1, lanes ULV-NO, GRA-NO,
 CHLO-NO, TETR-NO).

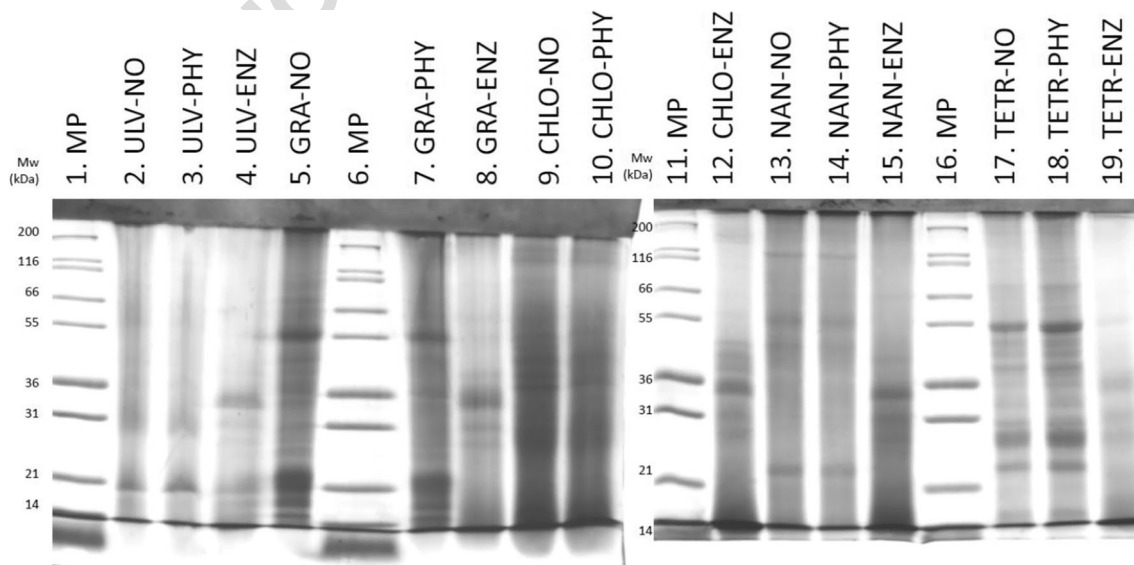


Fig. 1 SDS PAGE of the extracts of the fifteen algae ingredients included in this study ULV, *Ulva rigida*; GRA, *Gracilaria gracilis*; NAN, *Nannochloropsis oceanica*; CHLO, *Chlorella vulgaris*; TETR,

Tetraselmis sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed; MP, Mark12™ was used as protein molecular weight marker

386 CHLO-NO, NAN-NO, and TETR-NO). The pattern of ULV-
 387 NO consisted of mainly two characteristic bands between 14
 388 and 31 kDa, whereas the other four algae had more and better-
 389 defined bands distributed differently over most of the molec-
 390 ular weight range of the gels (14 to 200 kDa). The physical
 391 processing of the algae (Fig. 1: ULV-PHY, GRA-PHY,
 392 CHLO-PHY, NAN-PHY, and TETR-PHY) did not result in
 393 clear detectable changes in the protein profile of any of the
 394 five tested algae, compared with the no-processing groups.
 395 Contrarily, the enzymatic processing clearly changed the pro-
 396 tein profile of all algae species resulting in a decrease in bands
 397 of high molecular weight proteins and an increase in low
 398 molecular weight proteins presented in the gels (Fig. 1,
 399 ULV-ENZ, GRA-ENZ, CHLO-ENZ, NAN-ENZ and
 400 TETR-ENZ) documenting an efficient effect of the enzyme
 401 treatment. FPLC analyses of all fifteen alga ingredients
 402 (Fig. 2) showed that the physical processing (PHY) had minor
 403 effects on the selected algae, compared to the enzymatic; low
 404 molecular compounds (peptide bond < 12.3 kDa based on
 405 integration of baseline subtracted FPLC profiles) evidenced

a 1.5-fold increase in *U. rigida* and *C. vulgaris* and 1.3-fold
 increase in *G. gracilis* (Table 2). However, the enzymatically
 process (ENZ) resulted in not only pronounced changes of the
 peaks' profiles of all the algae, but also in a generalized in-
 crease of low-molecular weight compounds (mainly peptides
 < 12.3 kDa) in all algae. For *U. rigida*, *G. gracilis*, and
N. oceanica, this increase in peptides < 12.3 kDa was substan-
 tially higher (3.6, 2.5, and 2.3-fold increase, respectively;
 Table 2) than that perceived in either *C. vulgaris* (1.6-fold
 increase) or *Tetraselmis* sp. (2.1-fold increase).

The experimental diets, obtained by replacing 30% of the
 reference diet by each alga, had 39–51% protein, 9.3–12% fat,
 19–25 kJ g⁻¹, 19.4–27% EAAs (Tables 4 and 5), reflecting the
 high variation observed in the nutritional value of each algae
 species.

The apparent digestibility coefficients (ADC) of macro nu-
 trients, energy, and individual amino acids of the seaweed-rich
 diets fed to European seabass juveniles are reported in
 Table 6. The dry matter ADCs of the experimental diets varied
 between 38 and 67%, with diets containing *G. gracilis*

Fig. 2 Size exclusion chromatograms of aqueous extracts of the two seaweeds and three microalgae before and after processing. Eluting compounds were detected at 215 nm. Cytochrome c (CytC, 12.3 kDa), aprotinin (6.5 kDa) and triglycine (Gly3, 189 Da) were used as external standards for molecular weight. The largest molecule is eluted first from the column. mAU – milli absorbance units, higher mAU corresponds to larger amount of low molecular compounds absorbing at 215 nm (peptide bond). NO, not processed; PHY, physically processed; ENZ, enzymatically processed

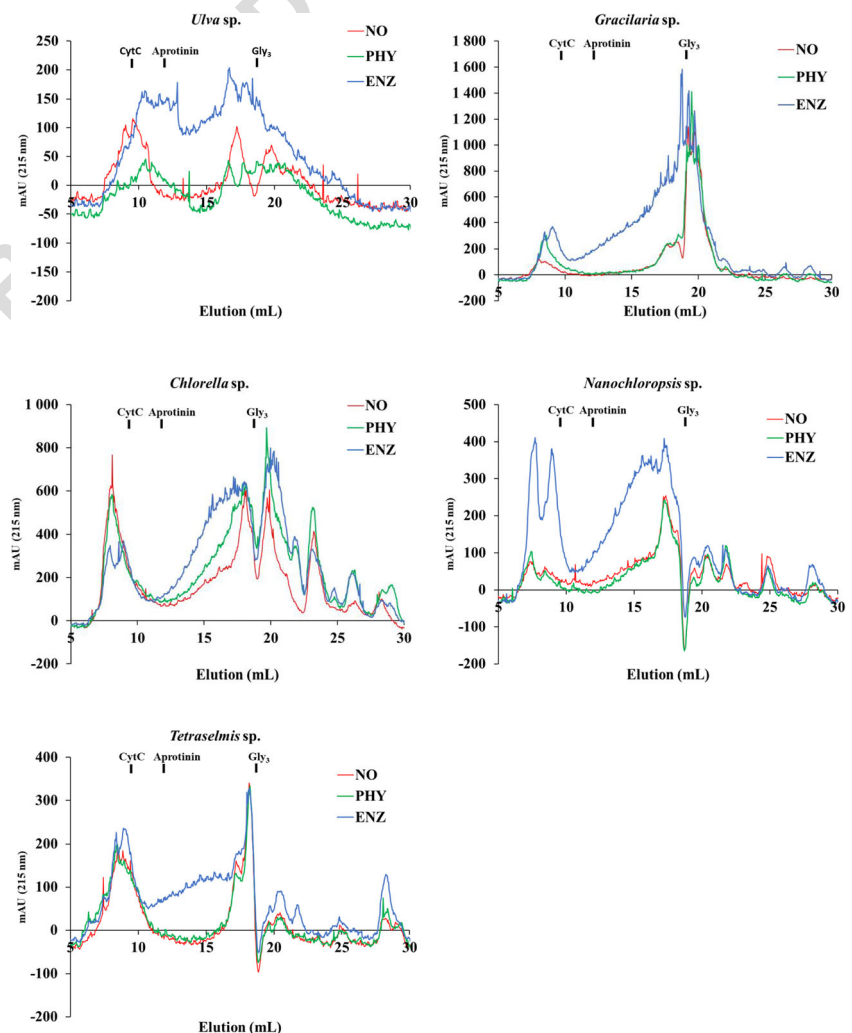


Table 6 Apparent digestibility coefficients (ADC) of nutrients and energy of the experimental diets containing seaweeds either used intact (NO) or after physic (PHY) or enzymatic (ENZ) processing

		ADC (%) of experimental diets							SEM	p value
		REF	DULV			DGRA				
			NO	PHY	ENZ	NO	PHY	ENZ		
t6.5	Dry matter	66.7 ^a	49.0 ^{bc}	38.2 ^c	41.7 ^c	60.0 ^{ab}	62.2 ^{ab}	60.2 ^{ab}	2.5	<0.001
t6.6	Protein	94.5 ^a	90.8 ^{ab}	87.2 ^b	87.4 ^b	93.2 ^a	93.9 ^a	92.7 ^a	0.7	<0.001
t6.7	Lipids	92.1 ^a	88.8 ^a	88.3 ^{ab}	79.6 ^b	94.3 ^a	91.5 ^a	91.9 ^a	1.2	0.002
t6.8	Energy	90.6 ^a	87.0 ^{ab}	85.8 ^b	86.0 ^b	86.1 ^{ab}	89.7 ^{ab}	88.6 ^{ab}	0.5	0.01
t6.9	EAA	95.6 ^a	93.1 ^{ab}	92.8 ^{ab}	91.8 ^b	93.7 ^{ab}	94.5 ^{ab}	93.6 ^{ab}	0.3	0.02
t6.10	Arginine	96.3	95.1	95.0	95.1	95.3	96.2	93.8	0.4	0.66
t6.11	Histidine	92.0	88.6	87.4	84.2	92.3	90.0	89.4	1.1	0.48
t6.12	Lysine	97.3 ^a	94.1 ^{ab}	94.5 ^{ab}	95.1 ^{ab}	93.5 ^{ab}	93.6 ^{ab}	93.0 ^b	0.4	0.03
t6.13	Threonine	95.2	92.9	92.9	92.6	93.6	93.9	93.9	0.3	0.05
t6.14	Isoleucine	95.1 ^a	91.3 ^{ab}	92.8 ^a	88.7 ^b	92.3 ^{ab}	94.7 ^a	93.2 ^a	0.5	0.002
t6.15	Leucine	96.3 ^a	94.3 ^{abc}	92.9 ^{bc}	91.7 ^c	95.0 ^{ab}	95.6 ^{ab}	95.0 ^{abc}	0.4	0.004
t6.16	Valine	94.2 ^a	90.9 ^{ab}	90.8 ^{ab}	89.3 ^b	92.3 ^{ab}	93.7 ^a	92.5 ^{ab}	0.4	0.003
t6.17	Methionine	98.3 ^{ab}	97.3 ^{bc}	95.3 ^d	96.4 ^{cd}	98.9 ^a	99.1 ^a	98.9 ^a	0.3	<0.001
t6.18	Phenylalanine	95.1 ^a	92.3 ^{ab}	92.0 ^c	91.0 ^b	91.6 ^b	92.7 ^{ab}	92.9 ^{ab}	0.3	0.01
t6.19	CEAA	97.0 ^a	95.0 ^b	94.2 ^b	94.3 ^b	95.1 ^b	95.8 ^{ab}	95.4 ^{ab}	0.2	0.002
t6.20	Cystine	98.3 ^a	97.9 ^a	ND	97.9 ^a	93.8 ^b	94.7 ^a	94.0 ^a	0.5	<0.001
t6.21	Hydroxyproline	96.9 ^a	92.1 ^b	89.7 ^b	91.2 ^b	96.5 ^a	96.6 ^a	96.4 ^a	0.7	<0.001
t6.22	Proline	96.7 ^a	94.9 ^{ab}	94.9 ^{ab}	94.1 ^b	95.1 ^{ab}	95.8 ^{ab}	95.5 ^{ab}	0.2	0.01
t6.23	NEAA	95.3 ^a	93.0 ^{ab}	92.7 ^b	92.9 ^b	94.7 ^{ab}	95.9 ^a	95.2 ^{ab}	0.3	0.01
t6.24	Alanine	95.0 ^a	91.6 ^{bc}	92.9 ^{abc}	90.7 ^c	93.9 ^{ab}	95.2 ^a	94.8 ^a	0.4	0.001
t6.25	Tyrosine	96.8 ^{ab}	94.5 ^{ab}	94.3 ^b	94.5 ^{ab}	96.1 ^{ab}	96.7 ^a	95.8 ^{ab}	0.3	0.02*
t6.26	Aspartate	94.9 ^a	91.4 ^{ab}	91.4 ^{ab}	90.3 ^b	93.9 ^{ab}	94.6 ^a	93.5 ^{ab}	0.4	0.004
t6.27	Glutamate	95.2 ^{ab}	93.8 ^{ab}	92.6 ^b	94.1 ^{ab}	95.5 ^{ab}	97.0 ^a	96.3 ^a	0.4	0.01
t6.28	Glycine	95.7 ^a	92.8 ^{ab}	93.9 ^b	93.8 ^{ab}	93.3 ^{ab}	94.4 ^a	94.4 ^{ab}	0.3	0.17
t6.29	Serine	95.6 ^a	93.3 ^{ab}	93.0 ^{ab}	92.1 ^b	94.0 ^{ab}	95.0 ^{ab}	94.2 ^{ab}	0.3	0.02

Values are presented as mean \pm SEM, $n = 3$. Values in the same row with different superscript letter differ significantly ($p < 0.05$). *without differences on post hoc test. ADC, apparent digestibility coefficient; REF, reference diet; DULV, diet with 30% *U. rigida*; DGRA, diet with 30%, *G. gracilis*; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

(DGRA) not differing significantly from the REF diet, but those with *U. rigida* (DULV) displaying significantly lower values. Protein and energy digestibility values were not affected by the dietary inclusion of *G. gracilis*, but were significantly reduced when processed *U. rigida* was included in the diets (DULV-PHY and DULV-ENZ). The ADC of lipids was not strongly affected by the dietary inclusion of seaweeds, although DULV-ENZ (80%) had a significantly lower ADC value compared to the REF diet. The amino acid ADC values were generally high (>90%) and followed the same trend reported for protein; diets including *G. gracilis* (DGRA) displayed similar values to the reference diet, but those including *U. rigida* showed decreased amino acid digestibility in particular the DULV-ENZ diet that have a significantly lower EAA ADC value compared to the REF diet.

The ADCs of the seaweeds are presented in Table 7. Overall, there was a significant effect of the tested seaweeds and technological process on nutrient digestibility, while the interaction of these factors was only significant in the case of lipid and methionine digestibility. *G. gracilis* was better digested by European seabass than *U. rigida*. Although the possessing technology had no significant impact on dry matter, protein, and energy ADC values, they increased by 19, 4, and 22%, in physically processed *G. gracilis* (GRA-PHY) in relation to the unprocessed algae. Contrarily, in *U. rigida*, the best ADC values were observed in unprocessed algae. The ADC of individual amino acids varied widely among algae and *G. gracilis* displayed the highest ADC values. The essential amino acids (EAA), conditionally essential amino acids (CEAA) and nonessential amino acids (NEAA) digestibility

Table 7 Apparent digestibility coefficients (ADC) of nutrients and energy of the tested seaweeds

t7.2	ULV			GRA			SEM	ANOVA			
	NO	PHY	ENZ	NO	PHY	ENZ		S	P	S × P	
t7.3											
t7.4	Dry matter	1.9 ^B	−35.1 ^B	−19.9 ^B	43.4 ^A	51.6 ^A	44.2 ^A	9.3	<0.001	0.52	0.24
t7.5	Protein	62.7 ^B	34.2 ^B	27.2 ^B	88.8 ^A	92.0 ^A	85.3 ^A	6.9	<0.001	0.07	0.09
t7.6	Lipids	0.5 ^a	11.4 ^a	−725.1 ^b	216.3 ^a	72.5 ^a	82.7 ^a	83.0	0.001	0.004	0.01
t7.7	Energy	71.3 ^B	64.7 ^B	65.3 ^B	71.2 ^A	86.9 ^A	81.5 ^A	2.7	0.01	0.69	0.13
t7.8	EAA	71.9 ^B	69.7 ^B	55.5 ^B	85.4 ^A	89.3 ^A	80.1 ^A	3.3	0.001	0.09	0.60
t7.9	Arginine	78.3	80.8	78.6	89.4	95.6	75.3	3.8	0.37	0.54	0.64
t7.10	Histidine	72.4	ND	ND	95.9	65.9 ^A	61.3	17.1	0.38	0.56	0.67
t7.11	Lysine	61.9	63.5	66.4	72.3	75.7	60.2	3.2	0.44	0.76	0.50
t7.12	Threonine	78.5 ^B	78.7 ^B	75.4 ^B	88.7 ^A	88.8 ^A	87.3 ^A	1.8	0.004	0.77	0.96
t7.13	Isoleucine	60.0 ^{Bxy}	73.0 ^{Bx}	28.1 ^{By}	82.2 ^{Axy}	93.0 ^{Ax}	81.0 ^{Ay}	5.8	<0.001	0.01	0.11
t7.14	Leucine	77.4 ^{Bx}	63.3 ^{Bxy}	43.6 ^{By}	89.1 ^{Ax}	91.9 ^{Axy}	85.1 ^{Ay}	4.6	<0.001	0.02	0.07
t7.15	Valine	70.9 ^B	70.7 ^B	55.2 ^B	85.7 ^A	91.7 ^A	83.0 ^A	3.4	<0.001	0.09	0.48
t7.16	Methionine	85.3 ^b	59.6 ^c	65.8 ^c	108.2 ^a	115.4 ^a	109.9 ^a	5.4	<0.001	0.03	0.001
t7.17	Phenylalanine	74.0 ^B	71.6 ^B	61.1 ^B	80.5 ^A	83.8 ^A	81.9 ^A	2.4	0.003	0.32	0.29
t7.18	CEAA	72.2 ^B	62.9 ^B	60.2 ^B	82.8 ^A	86.4 ^A	78.4 ^A	2.9	0.001	0.26	0.42
t7.19	Cystine	84.8	ND	71.4	78.8	78.9	71.0	3.9	0.76	0.56	0.79
t7.20	Hydroxyproline	78.5	69.5	70.2	ND	ND	ND	2.0	NA	0.11	NA
t7.21	Proline	74.8 ^B	74.4 ^B	61.2 ^B	84.7 ^A	89.1 ^A	82.1 ^A	2.8	0.003	0.14	0.55
t7.22	NEAA	77.7 ^B	74.3 ^B	72.6 ^B	91.8 ^A	98.2 ^A	94.3 ^A	3.1	<0.001	0.86	0.62
t7.23	Alanine	80.9 ^B	86.4 ^B	73.4 ^B	91.3 ^A	95.6 ^A	94.1 ^A	2.1	<0.001	0.07	0.13
t7.24	Tyrosine	80.0 ^B	79.5 ^B	70.9 ^B	94.0 ^A	96.5 ^A	90.0 ^A	2.6	<0.001	0.17	0.81
t7.25	Aspartate	66.4 ^B	67.8 ^B	57.4 ^B	90.1 ^A	93.3 ^A	80.9 ^A	3.8	<0.001	0.18	0.98
t7.26	Glutamate	81.7 ^B	62.5 ^B	80.9 ^B	97.9 ^A	108.3 ^A	111.8 ^A	5.2	0.001	0.50	0.30
t7.27	Glycine	70.2	79.7	76.9	81.9	87.4	85.1	2.4	0.07	0.44	0.93
t7.28	Serine	78.8 ^B	76.0 ^B	66.9 ^B	88.3 ^A	92.5 ^A	87.3 ^A	2.6	0.001	0.27	0.51

Values are presented as mean ± SEM, $n = 3$. Values in the same row with different superscript letter differ significantly ($p < 0.05$): differences among treatments (a, b); for a particular alga, differences caused by technological process (x, y); and for a particular technological process, differences caused by algae (A, B). ULV, *Ulva rigida*; GRA, *G. gracilis*; NO, not processed; PHY, physically processed; ENZ, enzymatically processed; ND, not determined; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. S, seaweed; P, process; EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

456 values of GRA-PHY were the highest, but without differing
457 significantly from GRA ($P = 0.09$). The enzymatic process
458 decreased the ability of seabass to digest leucine,
459 irrespectively of the seaweed included in the diet, while meth-
460 ionine was significantly better digested in non-processed
461 (ULV-NO) then in processed (ULV-PHY and ULV-ENZ)
462 *Ulva* sp.

463 The ADCs of nutrients, energy, and amino acids of the
464 experimental diets containing microalgae biomass are present-
465 ed in Table 8. The dry matter ADCs of the experimental diets
466 varied between 41 and 67%, with diets containing *N. oceanica*
467 (DNAN) or *C. vulgaris* (DCHLO) not differing significantly
468 from the REF diet. However, the dietary inclusion of
469 *Tetraselmis* sp. biomass, either unprocessed (DTETR-NO)
470 or enzymatically processed (DTETR-ENZ), resulted in a

471 significant decrease of dry matter ADC in relation to the
472 REF diet (41–50% vs 67%, respectively). The dietary inclu-
473 sion of unprocessed microalgae impaired protein ADC values,
474 but after technological processing, diets DNAN-ENZ (93%),
475 DCHLO-PHY (93%), and DCHLO-ENZ (92%) reached pro-
476 tein ADC values similar to those observed in the REF diet
477 (95%). Energy ADC in DNAN-ENZ, DTETR-PHY, and in
478 DCHLO diets did not differ from the REF diet. Lipid ADC
479 values were reduced in DNAN and DTETR diets, irrespectively
480 of the processing method, but not in diets containing
481 *C. vulgaris* (DCHLO). The amino acid ADC values were
482 generally above 90%, except for histidine in DCHLO-ENZ,
483 DTETR-NO, and DTETR-ENZ (> 83%). All CHLO diets had
484 significantly lower lysine ADC value (90%) than the REF diet
485 (97%). No differences were observed for total EAA and total

Table 8 Apparent digestibility coefficients (ADC) of nutrients and energy of the experimental diets containing microalgae either used intact (NO) or after physic (PHY) or enzymatic (ENZ) processing

		ADC (%) of experimental diets											
		REF	DNAN			DCHLO			DTETR			SEM	<i>p</i> value
			NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ		
t8.5	Dry matter	66.7 ^a	56.4 ^{ab}	62.9 ^{ab}	64.5 ^{ab}	59.0 ^{ab}	65.7 ^a	65.7 ^a	40.9 ^c	61.9 ^{ab}	49.9 ^{bc}	1.7	<0.001
t8.6	Protein	94.5 ^a	91.5 ^{bc}	91.2 ^{bc}	93.0 ^{ab}	91.6 ^{bc}	92.6 ^{ab}	92.3 ^{abc}	89.8 ^c	92.4 ^{bc}	90.8 ^{bc}	0.3	<0.001
t8.7	Lipids	92.1 ^a	85.0 ^{bc}	83.0 ^c	85.0 ^{bc}	90.4 ^a	90.5 ^a	89.5 ^{ab}	84.4 ^c	84.1 ^c	84.8 ^c	0.6	<0.001
t8.8	Energy	90.6 ^a	87.2 ^{bc}	87.2 ^{bc}	89.7 ^{ab}	88.0 ^{abc}	90.5 ^a	90.6 ^a	82.0 ^d	88.7 ^{abc}	86.0 ^c	0.5	<0.001
t8.9	EAA	95.6	93.9	94.0	93.7	92.9	93.8	93.1	91.9	94.2	93.5	0.2	0.11
t8.10	Arginine	96.3	94.0	95.9	95.1	95.5	95.0	95.1	93.9	95.4	96.1	0.3	0.48
t8.11	Histidine	92.0	92.0	90.3	89.7	89.3	91.3	83.3	83.3	90.4	87.2	1.1	0.56
t8.12	Lysine	97.3 ^a	94.8 ^{abc}	95.5 ^{ab}	94.9 ^{ab}	90.7 ^{bc}	89.0 ^c	90.1 ^{bc}	95.3 ^{ab}	94.0 ^{abc}	94.6 ^{abc}	0.6	0.001
t8.13	Threonine	95.2	92.8	93.9	93.5	93.3	94.2	93.3	91.4	93.7	93.8	0.2	0.05
t8.14	Isoleucine	95.1 ^a	94.1 ^{ab}	93.6 ^{ab}	93.3 ^{ab}	91.4 ^b	94.0 ^{ab}	93.7 ^{ab}	92.1 ^{ab}	94.9 ^a	92.6 ^{ab}	0.3	0.01
t8.15	Leucine	96.3 ^a	94.3 ^{abc}	94.2 ^{bc}	94.0 ^{abc}	94.0 ^{abc}	95.1 ^{ab}	94.3 ^{abc}	91.5 ^c	94.3 ^{abc}	93.2 ^{bc}	0.3	0.002
t8.16	Valine	94.2 ^a	93.2 ^a	92.5 ^a	92.2 ^a	91.4 ^{ab}	93.6 ^a	92.9 ^a	89.0 ^b	94.0 ^a	92.4 ^a	0.3	0.001
t8.17	Methionine	98.3	97.5	97.5	97.5	98.1	97.8	97.1	95.4	97.4	97.2	0.2	0.08
t8.18	Phenylalanine	95.1 ^a	93.1 ^a	93.4 ^a	93.9 ^a	93.0 ^{ab}	94.2 ^a	93.2 ^a	90.8 ^b	93.1 ^a	93.0 ^a	0.2	<0.001
t8.19	CEAA	97.0 ^a	96.3 ^a	96.2 ^a	96.1 ^a	95.9 ^a	96.5 ^a	96.1 ^a	94.0 ^b	96.1 ^a	95.3 ^{ab}	0.2	0.001
t8.20	Cystine	98.3 ^a	96.3 ^c	97.2 ^{bc}	97.4 ^{ab}	96.8 ^{bc}	97.0 ^{bc}	97.5 ^{ab}	96.3 ^c	97.4 ^{ab}	97.4 ^{ab}	0.1	<0.001
t8.21	Hydroxyproline	96.9 ^a	96.0 ^{ab}	94.3 ^{bc}	95.2 ^{ab}	95.8 ^{ab}	95.1 ^{ab}	95.8 ^{ab}	95.4 ^{ab}	95.4 ^{ab}	92.7 ^c	0.2	<0.001
t8.22	Proline	96.7 ^a	96.4 ^a	96.2 ^a	96.0 ^a	95.8 ^a	96.5 ^a	96.0 ^a	93.3 ^b	96.0 ^a	95.3 ^{ab}	0.2	0.001
t8.23	NEAA	95.3	94.3	95.0	94.6	93.8	95.3	94.1	93.5	95.0	94.5	0.2	0.27
t8.24	Alanine	95.0 ^a	94.2 ^a	94.0 ^a	94.3 ^a	93.3 ^a	94.7 ^a	93.5 ^a	89.8 ^b	94.7 ^a	94.2 ^a	0.3	0.001
t8.25	Tyrosine	96.8 ^a	95.2 ^{ab}	95.4 ^{ab}	95.5 ^{ab}	94.4 ^b	95.9 ^{ab}	95.2 ^{ab}	94.4 ^b	95.2 ^{ab}	95.6 ^{ab}	0.2	0.01
t8.26	Aspartate	94.9	93.6	94.1	93.6	93.2	94.3	93.0	92.6	94.5	92.7	0.2	0.19
t8.27	Glutamate	95.2	94.8	96.0	94.8	94.3	96.1	94.8	95.4	95.4	95.4	0.2	0.69
t8.28	Glycine	95.7 ^a	94.3 ^a	94.3 ^a	95.5 ^a	93.3 ^a	94.9 ^a	94.6 ^a	90.4 ^b	95.2 ^a	94.4 ^a	0.3	<0.001
t8.29	Serine	95.6 ^a	93.2 ^{ab}	94.2 ^{ab}	94.3 ^a	93.6 ^{ab}	94.5 ^{ab}	93.3 ^{ab}	91.9 ^b	94.9 ^a	93.8 ^{ab}	0.2	0.02

Values are presented as mean \pm SEM, $n = 3$. Values in the same row with different superscript letter differ significantly ($p < 0.05$). ADC, apparent digestibility coefficient; REF, reference; DNAN, diet with 30% *N. oceanica*; DCHLO, diet with 30% *C. vulgaris*; DTETR, diet with 30% *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

486 NEAA ADCs, between the different dietary treatments.
487 However, CEAA ADC in diet DTETR-NO was significantly
488 lower (94%) than the REF diet (97%).

489 When considering the digestibility of microalgae (Table 9),
490 overall, a significant effect of the tested seaweeds, technolog-
491 ical process, and interaction of both factors on nutrient digest-
492 ibility was observed. *Chlorella vulgaris* and *N. oceanica* gen-
493 erally had higher nutrient ADC values compared to
494 *Tetraselmis* sp. Unprocessed microalgae had the lowest nutri-
495 ent and energy digestibility values. Technological processing
496 irrespective of the method applied, significantly ($p < 0.05$) im-
497 proved microalgae dry matter digestibility (> 50% increase).
498 The highest protein ADC values were registered in NAN-
499 ENZ, CHLO-PHY, and CHLO-ENZ (> 88%), showing an

500 increase of 8, 4, and 3%, respectively, in relation to their
501 unprocessed counterparts. The highest increase in protein
502 ADC was observed in *Tetraselmis* sp. after physical process-
503 ing (TETR-PHY, 20% increase). Technological processing
504 dramatically enhanced energy ADC values in relation to un-
505 processed algae: 14% increase in NAN-ENZ; 11% in both
506 CHLO-PHY and CHLO-ENZ; 66% in TETR-PHY and 40%
507 in TETR-ENZ. The highest energy ADC values were ob-
508 served in CHLO-PHY and CHLO-ENZ (> 90%).
509 *Tetraselmis* sp. had the lowest energy (49%) ADC's, which
510 was significantly enhanced ($p < 0.05$) after the physical pro-
511 cess (66% increase, in relation to the unprocessed
512 microalgae). Lipid ADC values of *Tetraselmis* sp. were sig-
513 nificantly lower than the other microalgae and were extremely

t9.1 **Table 9** Apparent digestibility coefficients (ADC) of nutrients and energy of the tested microalgae

t9.2	NAN			CHLO			TETR			SEM	ANOVA			
	NO	PHY	ENZ	NO	PHY	ENZ	NO	PHY	ENZ		M	P	M × P	
t9.4	Dry matter	32.0 ^{Ay}	53.6 ^{Ax}	59.4 ^{Ax}	41.2 ^{Ay}	63.4 ^{Ax}	63.4 ^{Ax}	-19.1 ^{By}	51.4 ^{Bx}	12.5 ^{Bx}	5.8	<0.001	0.001	0.09
t9.5	Protein	81.6 ^{ab}	81.0 ^{abc}	87.9 ^a	85.5 ^{ab}	88.6 ^a	87.6 ^a	69.7 ^c	83.6 ^{ab}	73.7 ^{bc}	1.4	<0.001	0.03	0.02
t9.6	Lipids	63.1 ^a	56.1 ^a	63.8 ^a	84.9 ^a	81.2 ^a	78.4 ^a	-92.4 ^b	-101.2 ^b	-795.0 ^c	52.9	<0.001	<0.001	<0.001
t9.7	Energy	76.2 ^{bc}	76.6 ^{abc}	87.0 ^{ab}	81.5 ^{abc}	90.4 ^a	90.6 ^a	48.9 ^d	81.1 ^{abc}	68.3 ^c	2.5	<0.001	<0.001	<0.001
t9.8	EAA	88.7 ^A	89.2 ^A	87.2 ^A	86.9 ^{AB}	90.1 ^{AB}	87.2 ^{AB}	74.3 ^B	88.1 ^B	82.7 ^B	1.2	0.03	0.10	0.27
t9.9	Arginine	86.1	94.6	90.6	93.2	92.2	92.8	81.4	90.9	95.0	1.3	0.49	0.13	0.33
t9.10	Histidine	92.0	83.6	78.8	74.4	88.2	50.3	59.4	78.7	49.3	6.6	0.46	0.38	0.93
t9.11	Lysine	87.5 ^A	90.5 ^A	86.4 ^A	73.8 ^B	71.7 ^B	73.8 ^B	84.2 ^{AB}	76.2 ^{AB}	76.8 ^{AB}	1.9	0.01	0.77	0.83
t9.12	Threonine	86.2 ^{ABy}	90.5 ^{ABx}	88.7 ^{ABxy}	90.3 ^{Ay}	92.3 ^{Ax}	89.0 ^{Axy}	74.8 ^{By}	87.8 ^{Bx}	87.5 ^{Bxy}	1.2	0.01	0.02	0.10
t9.13	Isoleucine	90.5 ^{abc}	88.4 ^{abc}	86.7 ^{abc}	83.4 ^{abc}	91.9 ^{ab}	89.7 ^{abc}	76.5 ^c	93.9 ^a	78.0 ^{bc}	1.4	0.04	0.01	0.02
t9.14	Leucine	88.5 ^a	88.1 ^{ab}	86.0 ^{ab}	89.1 ^a	92.6 ^a	89.2 ^a	71.1 ^c	85.6 ^{ab}	77.4 ^{bc}	1.4	<0.001	0.01	0.04
t9.15	Valine	91.1 ^a	88.6 ^a	87.5 ^a	86.7 ^a	92.7 ^a	90.5 ^a	69.1 ^b	93.3 ^a	86.1 ^a	1.5	0.004	0.001	<0.001
t9.16	Methionine	61.4	ND	91.2	97.1	95.8	67.3	73.9	89.9	86.5	3.7	0.77	0.24	0.04*
t9.17	Phenylalanine	87.4 ^{Ay}	88.4 ^{Ax}	89.9 ^{Axy}	88.7 ^{Ay}	92.3 ^{Ax}	88.3 ^{Axy}	74.4 ^{By}	85.0 ^{Bx}	83.7 ^{Bxy}	1.1	<0.001	0.01	0.06
t9.18	CEAA	93.1 ^a	92.0 ^a	91.3 ^a	91.9 ^a	94.5 ^a	92.2 ^a	68.9 ^b	89.8 ^a	81.7 ^{ab}	1.7	<0.001	0.01	0.01
t9.19	Cystine	ND	ND	ND	90.5 ^a	91.8 ^a	87.3 ^a	42.5 ^b	77.8 ^a	80.7 ^a	4.2	<0.001	<0.001	<0.001
t9.20	Hydroxyproline	ND	ND	ND	35.6	ND	ND	ND	46.3	ND	7.4		0.53**	
t9.21	Proline	95.1 ^a	94.1 ^a	93.1 ^a	93.0 ^a	95.9 ^a	93.4 ^a	71.8 ^b	92.2 ^a	86.1 ^a	1.6	<0.001	0.01	0.01
t9.22	NEAA	90.4 ^{Ay}	93.6 ^{Ax}	91.3 ^{Axy}	90.5 ^{Ay}	95.2 ^{Ax}	90.6 ^{Axy}	82.1 ^{By}	93.6 ^{Bx}	89.6 ^{Bxy}	1.0	0.24	0.04	0.55
t9.23	Alanine	92.4 ^a	91.8 ^a	92.7 ^a	91.5 ^a	94.4 ^a	91.0 ^a	74.3 ^b	93.7 ^a	91.7 ^a	1.3	0.01	0.003	0.001
t9.24	Tyrosine	89.3 ^A	90.0 ^A	90.0 ^A	91.0 ^A	94.6 ^A	89.6 ^A	81.6 ^B	86.8 ^B	84.5 ^B	0.9	<0.001	0.13	0.55
t9.25	Aspartate	89.2 ^y	91.5 ^x	88.2 ^y	89.4 ^y	93.0 ^x	87.7 ^y	77.7 ^y	92.4 ^x	81.2 ^y	1.3	0.04*	0.02	0.34
t9.26	Glutamate	93.1	99.5	92.6	91.8	99.0	93.5	98.0	96.9	97.2	1.3	0.76	0.38	0.88
t9.27	Glycine	89.9 ^a	89.9 ^a	94.8 ^a	87.6 ^a	92.8 ^a	91.7 ^a	68.3 ^b	93.1 ^a	88.1 ^a	1.6	<0.001	<0.001	<0.001
t9.28	Serine	82.4 ^{ab}	87.9 ^a	87.8 ^a	89.1 ^a	91.7 ^a	84.9 ^{ab}	67.3 ^b	91.0 ^a	81.2 ^{ab}	1.7	0.02	0.01	0.04

Values are presented as mean ± SEM, n = 3. Values in the same row with different superscript letter differ significantly (p < 0.05): differences among treatments (a, b); for a particular alga, differences caused by technological process (x, y); and for a particular technological process, differences caused by alga (A, B). *without differences on post hoc test. **One-way ANOVA. NAN, *N. oceanica*; CHLO, *C. vulgaris*; TETR, *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. M, microalgae; P, process; EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

514 negative. Concerning individual EAA amino acids, it was
 515 observed that *N. oceanica* had the highest lysine ADC value,
 516 followed by *Tetraselmis* sp. and *C. vulgaris*. Threonine, phe-
 517 nylalanine, and aspartate's digestibility was significantly im-
 518 proved (p < 0.05) in all microalgae after physical processing.
 519 EAA ADC values were not significantly affected by the tech-
 520 nological processing.

521 **Discussion**

522 The nutritional value of an ingredient for a certain fish species
 523 depends on its chemical composition but also on the bioavail-
 524 ability of its nutrients and energy, and this can be evaluated by
 525 their apparent digestibility coefficients, ADCs (NRC 2011).

To date, there is still few information concerning ADC values
 for most of the algae species that are emerging as possible
 ingredients for aquafeeds and this is a major step towards
 the formulation of nutritionally balanced diets for any fish
 species. The nutritional value of the algae used in this study
 was very variable among species, with *C. vulgaris* having the
 highest protein content (54%) followed by *N. oceanica* and
G. gracilis (35%). *C. vulgaris* and *N. oceanica* had higher
 content of essential amino acids, EAA (> 20%), and were
 characterized by a high lipid content (10%), indicating that
 they could be good quality protein and lipid sources for
 aquafeeds. The inorganic matter (ash) was highest (> 30%)
 in *N. oceanica*, followed by *Tetraselmis* sp. and *U. rigida*,
 and these algae were particularly rich in Na, K, and Mg. The
 nutrient composition of both micro- and macroalgae has been

541 reported in literature and values vary greatly among species,
542 cultivation strategies, seasons, and locations (Makkar et al.
543 2016; Neto et al. 2018; Tibbetts 2018), evidencing the need
544 for an adequate nutritional evaluation of each lot prior use in
545 aquafeeds. At the same time, the composition of farmed algae,
546 as those used in this study, is rather consistent and their nutri-
547 tional profile can be customized to meet the needs of the end
548 product.

549 In this study physical-mechanical and enzymatic techno-
550 logical processes were applied to the no-processed algae to
551 disrupt cell walls and promote the accessibility of intracellular
552 nutrients. The physical-mechanical processing of the algae did
553 not result in clear detectable changes in the protein bands
554 profile of any of the five algae species as could be perceived
555 by SDS PAGE, but when analyzed by size exclusion chroma-
556 tography in the FPLC evidenced increased amount of low
557 molecular compounds (peptide and amino acids) mainly in
558 *U. rigida* and *C. vulgaris*. Moreover, the enzymatic process-
559 ing clearly changed the protein profile of all algae, decreasing
560 high-molecular-weight proteins and increasing the amount of
561 low-molecular-weight ones. Both SDS PAGE and FPLC anal-
562 ysis evidenced that the enzymatic process was more effective
563 than the physical-mechanical in changing the protein and pep-
564 tides composition of the different algae, resulting in a partic-
565 ularly relevant increase of low-molecular-weight compounds
566 especially in *U. rigida*, *G. gracilis*, and *N. oceanica*. Previous
567 reports have shown that conventional mechanical and enzy-
568 matic methods for protein extraction may affect the integrity
569 of extracted algal proteins due to the release of proteases from
570 cytosolic vacuoles (Bleakley and Hayes 2017). Such intrinsic
571 proteases could be partly responsible for the reduced presence
572 of high-molecular-weight proteins after enzymatic processing
573 as observed in the present study, especially for the *U. rigida*,
574 *G. gracilis*, and *N. oceanica*, resulting in larger amount of low
575 molecular compounds absorbing at 215 nm (peptide and ami-
576 no acids) after enzymatic processing. These results are in gen-
577 eral accordance with previous observations by Fleurence et al.
578 (1995) reporting improved protein solubilization from edible
579 seaweeds after the combined action of a polysaccharidase
580 mixture (agarase and cellulase). Moreover, using a simulated
581 in vitro gastrointestinal digestion model, Maehre et al. (2016)
582 showed that enzymatic pre-treatment of seaweed biomass re-
583 sulted in a 3-fold increase in amino acids available for intes-
584 tinal absorption and could thus be an effective method for
585 increasing the utilization potential of seaweed proteins. Nutrient
586 accessibility was previously shown to play an impor-
587 tant role in the nutrient digestibility in microalgae (Teuling
588 et al. 2019), but this has to be confirmed by in vivo digestibil-
589 ity trials with target species.

590 In the present study, the formulation of the test diets follow-
591 ed a classic approach and was obtained by replacing 30% of a
592 reference diet (REF) by the algal biomass, either before (en-
593 tire, not processed algae, NO) or after physical-mechanical

(PHY) or enzymatic (ENZ) processing. The composition of 594
each test diet largely reflected the composition of each algae 595
resulting in a quite imbalanced composition (crude protein 596
varying between 39 and 51% DM, crude fat between 9 and 597
14% DM, and gross energy between 19 and 25 kJ g⁻¹ DM). 598
This is not an optimal approach but is the most widely used 599
and accepted in nutritional trials (NRC 2011). According to 600
our knowledge, very few studies evaluated the digestibility of 601
either microalgae (Safari et al. 2016; Sarker et al. 2016; 602
Tibbetts et al. 2017; Gong et al. 2018; Agboola et al. 2019; 603
Teuling et al. 2019) or seaweeds (Pereira et al. 2012) in fish 604
species, and in European seabass, studies are even scarcer 605
(Valente et al. 2019a). 606

607 In our study, the dry matter, protein, and energy digestibil-
608 ity of the test diets containing *G. gracilis* did not differ from
609 the REF diet, but the dietary inclusion of *U. rigida* negatively
610 affected dry matter ADC values. *U. rigida*, when included at
611 such high dietary inclusion level (30%), seems to have a lower
612 nutritional value associated with its chemical composition and
613 bioavailability of nutrients. The dry matter ADC reflects the
614 digestible fraction of both organic and inorganic matter and is
615 largely dependent on its insoluble carbohydrates and mineral
616 composition. *Ulva rigida* has not only higher ash content but
617 also a higher content of high-molecular-weight proteins com-
618 pared to *G. gracilis* that may have contributed to the lower dry
619 matter ADC value. Moreover, the complexity of algal poly-
620 saccharides in seaweeds may also have contributed to ob-
621 served differences in digestibility and merits further evalua-
622 tion. The lipid digestibility values presently reported for both
623 seaweeds were highly variable and in some case might be
624 considered an artifact probably due to the very low lipid con-
625 tent of the seaweeds (0.6–1.2%). There are no previous studies
626 focused on the digestibility of seaweeds in European seabass,
627 but Pereira et al. (2012) evaluated the ADCs of four different
628 seaweeds, including *Ulva* spp. and *G. vermiculophylla* in rain-
629 bow trout (*Onchorynchus mykiss*) and Nile tilapia
630 (*Oreochromis niloticus*). For both fish species, the dry matter
631 ADC of the experimental diets was lower than that of the
632 reference diet, but in rainbow trout, protein and energy digest-
633 ibility were highest in *G. vermiculophylla*. Likewise, the present
634 results showed that *G. gracilis* is better digested by
635 European seabass than *U. rigida*. In fact, there was a signifi-
636 cant effect of the tested seaweeds and technological process
637 on nutrient digestibility. Dry matter ADC was increased by
638 19% in physically processed *G. gracilis* (GRA-PHY), contrib-
639 uting to a 22% increase in the energy ADC value. The EAA,
640 CEAA, and NEAA digestibility values of GRA-PHY were the
641 highest and contributed to the 4% increase in protein ADC.
642 Although the increased ADC values of GRA-PHY were not
643 significantly different from GRA-NO, we should keep in
644 mind that this is a very short-term digestibility trial, so the
645 dietary inclusion of this ingredient in a longer-term growth
646 trial merits further consideration. Contrarily, both physical

647 and enzymatic processing technologies had a negative impact
 648 on *U. rigida* nutrient digestibility. Although the enzymatic
 649 process seems to be effective in increasing low-molecular-
 650 weight proteins, it might also have released complex polysac-
 651 charides that impaired nutrient digestibility. According to the
 652 literature, green algae cell wall is mainly constituted by poly-
 653 saccharides (up to 54% of the algae dry weight) comprising
 654 both insoluble (cellulose, hemicelluloses, and lignin) and
 655 water-soluble sulphate polysaccharides, ulvan (8–29%).
 656 Ulvan seems to have an atypical gelling mechanism that
 657 may interfere with biological functions that are yet to be iden-
 658 tified (Lahaye and Robic 2007). The negative value observed
 659 for dry matter digestibility, after *U. rigida* technological pro-
 660 cessing, suggests an antagonistic property of the test ingredi-
 661 ent for the absorption of nutrients. This was particularly evi-
 662 dent in some essential amino acids like leucine and methio-
 663 nine in which the digestibility was significantly reduced in
 664 processed *U. rigida*. In the case of methionine, a significant
 665 interaction was observed between the algae strain and the
 666 technological processing. But overall, results suggest that the
 667 tested processing methodologies do not seem to be appropri-
 668 ate to this alga species before its inclusion in diets for
 669 European seabass.

670 The dry matter digestibility of *N. oceanica* biomass in the
 671 test diets did not differ from the REF diet, but protein, lipid,
 672 and energy ADCs were significantly reduced in unprocessed
 673 algae (DNAN-NO). Likewise, in a digestibility study with
 674 Atlantic salmon, Gong et al. (2018) reported impaired protein
 675 (82 vs 86%) and energy (77 vs 83%), but not dry matter ADC
 676 values (67 vs 69%) in extruded diets with 30% defatted
 677 *Nannochloropsis* sp., compared to the reference diet.
 678 Untreated *Nannochloropsis gaditana*, also in a digestibility
 679 study, resulted in decreased dry matter, protein, lipid, and
 680 energy ADC values, in both African catfish (Agboola et al.
 681 2019) and Nile tilapia (Teuling et al. 2019) compared to the
 682 reference diet. The only digestibility study performed in
 683 European seabass reported dry matter and protein ADCs of
 684 68 and 85%, respectively, for defatted *Nannochloropsis* sp.
 685 (Valente et al. 2019a). These values are higher than those
 686 presently observed for no-processed *N. oceanica* but within
 687 the range of values observed for NAN-ENZ. The higher pro-
 688 tein and lower fat content of defatted biomass, together with a
 689 possible positive effect of the defatting process on nutrient
 690 bioavailability may explain such differences. In the present
 691 study, and contrarily to seaweeds, a significant effect of the
 692 tested seaweeds, technological process, and interaction of
 693 both factors on nutrient digestibility was observed. Dry matter
 694 ADC more than doubled in both NAN-PHY and NAN-ENZ,
 695 but protein and energy ADCs have only increased with enzy-
 696 matic processing (88 vs 82% and 87 vs 76%, respectively).
 697 These results suggest a higher effectiveness of enzymatic cell
 698 wall disruption to increase bioavailability of *N. oceanica* nu-
 699 trients which is generally in accordance with the SDS PAGE

700 and FPLC data. The profiles of low molecular compounds
 701 from unprocessed and physically processed *N. oceanica* have
 702 high similarity, but when an enzymatic hydrolysis is applied
 703 to *N. oceanica*, the amount of low molecular compounds in-
 704 creased substantially. As an example, the amount of low mo-
 705 lecular compounds, between 6.5 kDa (aprotinin) and 189 Da
 706 (Gly3), almost tripled. Moreover, increased soluble protein
 707 was reported in *Nannochloropsis* sp. after enzymatic hydroly-
 708 sis (Valente et al. 2019b), which may partially explain the
 709 increased protein ADC presently observed. The digestibility
 710 of EAA was not significantly affected by the technological
 711 process (> 87%), but threonine and phenylalanine ADCs sig-
 712 nificantly increased in NANO-PHY. Curiously, the enzymatic
 713 process of *N. oceanica* has simultaneously increased the
 714 amount of peptides in the high-molecular end of the analysis,
 715 despite still being classified as low-molecular compounds
 716 (less than 20–30 kDa). We may hypothesize that this is a
 717 result of protein/peptide aggregation due to polysaccharide
 718 release, due to the release of proteins from the cell wall and/
 719 or cleavage of bigger (maybe insoluble) proteins into soluble
 720 peptides, and due to the action of cellulases. In any case, this
 721 might have increased nutrient accessibility and ultimately lead
 722 to increased protein and energy ADC values. The presence of
 723 intact cell wall seems a limiting factor for *Nannochloropsis* sp.
 724 digestibility in several fish species. Different cell wall disrup-
 725 tion methods were used to increase bioavailability of
 726 *N. gaditana* nutrients for Nile tilapia, showing that bead mill-
 727 ing the algae increased protein (78 vs 62%) ADC values in
 728 ingredient level, which were positively correlated with nutri-
 729 ent accessibility determined in vitro (Teuling et al. 2019).
 730 Moreover, in Atlantic salmon, extrusion processing signifi-
 731 cantly increased *Nannochloropsis* sp. dry matter ADC com-
 732 pared to cold-pelleting, but protein ADC remained unaffected
 733 (Gong et al. 2018).

734 The dry matter digestibility of *C. vulgaris* biomass in the
 735 test diets did not differ from the REF diet, but protein ADC
 736 was significantly reduced in unprocessed algae, evidencing
 737 the importance of cell wall disruption to improve nutrients
 738 digestibility. In fact, dry matter digestibility of *C. vulgaris* as
 739 single ingredient more than doubled in processed algae and
 740 protein ADC values increased 4% in CHLO-PHY (89 vs
 741 86%) compared to unprocessed algae. The FPLC profiles
 742 did not reveal pronounced differences between processes ap-
 743 plied to *C. vulgaris*, but an increase of low molecular com-
 744 pounds can be clearly observed in both technological process-
 745 es and resulted in the highest protein ADC value for this spe-
 746 cies. Moreover, CHLO-PHY had generally high digestibility
 747 values for individual EAA, with threonine and phenylalanine
 748 ADCs having significantly higher ADC values than those ob-
 749 served for unprocessed algae (CHLO-NO). As far as we
 750 know, the digestibility of *C. vulgaris* has never been evaluated
 751 in European seabass, but in Atlantic salmon, previous studies
 752 demonstrated that dry matter, protein, lipid, and energy

digestibility dropped off in a relatively dose-dependent manner with the dietary inclusion of whole cell meal (Tibbetts et al. 2017). However, Tibbetts et al. (2017) have also shown that cell-rupture *C. vulgaris* biomass (by microfluidics), when included at 30%, could only significantly improve digestibility of dry matter and carbohydrates. This resulted in a protein ADC value of 85% for processed alga, which compares well with the present result for unprocessed *C. vulgaris* but is lower than values observed for either CHLO-PHY (89%) or CHLO-ENZ (88%). This difference may be explained by the comparatively higher protein and lower lipid content of the algal biomass used in our trial (54 vs 30% and 10 vs 26%, respectively). In fact, Tibbetts et al. (2017) predominantly related to the reduction of energy digestibility in Atlantic salmon fed 30% disrupted *C. vulgaris* to the dietary lipid fraction. But this could not be confirmed in our study as lipid and energy ADCs remained unaffected by the technological processing. In Nile tilapia, Sarker et al. (2016) reported a protein ADC of 80% for *Chlorella* sp. which is lower than the value presently reported for the unprocessed algae (86%) in spite of its equivalent biochemical composition. Authors attributed the low nutrient and energy ADC of *Chlorella* sp. to its high fiber content that might have inhibited proteolytic enzymatic activity. However, the present results evidenced the effectiveness of both the physical and the enzymatic processing of this microalga in improving protein and energy ADC, resulting in the highest values in CHLO-PHY and CHLO-ENZ.

Among tested algae, no-processed *Tetraselmis* sp. had the lowest protein (70%) and energy (49%) digestibility coefficients. The genus *Tetraselmis* is unique among the green algae in its cell wall formation; its cell body is covered by a solid cell wall (theca), formed by extracellular fusion of scales mainly composed of acidic polysaccharides (Arora 2016). In fact, SDS PAGE and FPLC results revealed limited differences in the amount of low molecular compounds between processed and unprocessed *Tetraselmis* sp., evidencing the strong resistance of these microalgae to disruption. However, the physical process of these microalgae was able to significantly improve protein and energy ADCs values by 20% and 66%, respectively. The digestibility of EAA was also significantly enhanced in processed *Tetraselmis* sp. (11–19% increase). This effect was particularly relevant in TETR-PHY that resulted in increased digestibility of threonine, isoleucine, leucine, valine, and phenylalanine with values above 85%. The negative lipid ADC values of *Tetraselmis* sp. stands out from the rest microalgae. This could either be an artifact resulting from the low lipid level of these algae, or could be associated to the high resistance of its cell wall structure to the digestive enzymes, which may inhibit lipid digestion. Tuelling et al. (2019) reported a significantly high correlation between fat ADC and hydrolysis degree ($r = 0.94$), while Bitou et al. (1999) demonstrated that many marine algae inhibited the activity of pancreatic lipase. According to the literature, the

digestibility of *Tetraselmis* sp. has never been evaluated in fish as single ingredient, but a linear decline in nutrient digestibility was observed in European seabass fed diets with increasing levels of *Tetraselmis suecica* (Tulli et al. 2012). These results evidenced the difficulty of fish to access nutrients of this microalga, highlighting the need of technological processes prior its inclusion in aquafeeds. ADC values presently observed for several individual amino acids were significantly improved after physical technological processing of *Tetraselmis* sp., and in many cases with a significant interaction between tested seaweed and technological process. These results evidenced not only the efficiency of the alga processing in improving nutrient digestibility but also the need to select the most adequate method to disrupt the cell wall of each species.

In conclusion, the ability of European seabass to digest algae depends both on the selection of the most adequate algae species and on their technological processing. *Gracilaria gracilis* is better digested by seabass than *U. rigida*, and GRA-PHY merits further evaluation in long-term trials as resulted in the highest dry matter, protein, and energy ADCs. *Nnannochloropsis oceanica* and *C. vulgaris* are better digested than *Tetraselmis* sp., and contrarily to seaweeds, their technological processing significantly affected nutrient digestibility. Protein and energy ADCs were highest in NAN-ENZ and CHLO-PHY, followed by TETR-PHY. Results clearly showed that it is possible to increase nutrient accessibility and digestibility of algae for European seabass, by selecting the most adequate method to disrupt the cell wall. It is also important to mention that, unlike many other experimental cell rupture methods reported in literature, the physical-mechanical and enzymatic technological processes used in this study are scalable to industrial level. Further studies are warranted to evaluate the potential of using such processed algae biomasses during long-term growth trials to fully address their potential as ingredients for aquafeeds.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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