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Physical processing or supplementation of feeds with phytogenic compounds, alginate oligosaccharide or nucleotides as methods to improve the utilization of *Gracilaria gracilis* by juvenile European seabass (*Dicentrarchus labrax*)

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Running title: *Gracilaria* sp. as ingredients for European sea bass diets.

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Abstract

This study assessed both the effectiveness of a physical-mechanical rupture method and the ability of feed additives (phytogenic compounds, alginate oligosaccharide and nucleotides) to enhance the utilization of *G. gracilis* by European seabass. A commercial-based diet was used as control diet (CTRL) and compared with five isoproteic (53.5% Dry matter, DM) and isolipidic (14.9% DM) diets containing 8% of *G. gracilis*. This seaweed was either unprocessed (diet GRA) or subjected to physical processing (diet GRAP). The three additive-containing diets were formulated by supplementing the GRA diet with either 0.02% phytogenic compounds (PHY), 2.5% oligo-alginate (OAS) or 0.08% free nucleotides (NUC). Triplicate groups of nineteen fish (29.7 ± 0.02 g) were distributed by 50 L tanks (11.3 kg m^{-3}) and fed the experimental diets to satiety during 106 days. By the end of the trial, growth performance and nutrient utilization (specific growth ratio, feed conversion ratio, apparent digestibility coefficients, nutrient balance, intestinal brush border membrane enzyme activities and plasma metabolic parameters), gut histomorphology, antioxidant and immunological status of fish were evaluated. The ability of fish to digest seaweed-rich diets was largely improved by the technological processing of *G. gracilis*, albeit nil effect on fish specific growth rate (1.0 in all groups). This major achievement was associated with increased ability of GRAP to digest protein (84 *vs* 68% in GRA) and energy (64 *vs* 38% in GRA). The use of feed additives in *Gracilaria*-rich diets was less efficacious in improving European sea bass nutrient and energy ADCs, but have still improved the overall digestibility of those diets. Fish fed alginate oligosaccharide was mainly associated with increased activity of anterior intestine enzymes, particularly intestinal alkaline phosphatase (IAP; 174.4 *vs* 104.7-120.6 µm min^{-1} g^{-1} in *Gracilaria*-rich diets). Moreover, the algae technological processing and both the nucleotides and the alginate oligosaccharide seem to have positively affected the intestinal *villus* width compared to the negative impact seen in fish fed GRA. The tested additives had limited impact on oxidative stress, although glutathione peroxidase (GPx; 2.1 µmol min^{-1} mg $protein^{-1}$) and catalase (CAT; 35 µmol min^{-1} mg $protein^{-1}$) activities were lowest in fish fed NUC and PHY, respectively. It can be concluded that the physical processing of *Gracilaria* sp. or the addition of either oligo-alginate or nucleotides can effectively increase the nutritional value of this seaweed for European seabass diets.

Keywords: algae nutrient bioavailability; cell disruption methods; feed additives; intestinal morphology; digestive enzymes

1. Introduction

Future growth of the aquaculture sector is greatly dependent on the adoption of sustainable practices. Among them incorporation of alternative protein and lipid sources in aquafeeds will reduce the dependency on fishmeal and fish oil that are currently obtained from wild fish stocks. In this context, seaweeds can be regarded as valuable natural sources of nutrients and bioactive compounds in aquafeeds. Global aquaculture production of seaweeds has increased in recent years, mostly in Asia, and reached almost 30 million tonnes in 2015 (FAO, 2018). *Gracilaria spp.* are among the world's most cultivated and valuable edible seaweeds. They can achieve the high biomass yields required for the production of agar, and they find use for bioremediation of fish farm effluents (Abreu et al., 2011; Buschmann et al., 2017; Capo et al., 1999; Oliveira et al., 2000). *Gracilaria* sp. are natural sources of high-quality protein, minerals, bioactive compounds and functional polysaccharides (Angell et al., 2016; Holdt and Kraan, 2011). Several studies have evaluated the dietary inclusion of *Gracilaria* sp. in fish diets, perceiving them either as replacers of fish meal (Araújo et al., 2016; Batista et al., 2020b; Silva et al., 2015; Valente et al., 2006; Vizcaíno et al., 2016; Younis et al., 2018) or as supplements (<5%) to modulate immunological status, oxidative stress response, or fillet properties (Magnoni et al., 2017; Peixoto et al., 2019a; 2019b). Low digestibility and growth impairment are aspects that still hamper their inclusion at higher levels i.e. above 5-10%. Silva et al. (2015) observed a significant reduction in *villi* length, intestine diameter and increased feed conversion ratio along with growth impairment in Nile tilapia *Oreochromis niloticus* fed *G. vermiculophylla*. Likewise, in rainbow trout *Oncorhynchus mykiss* decreased intestinal absorption area was reported in fish fed diets containing above 9% *Gracilaria sp.* (Araújo et al., 2016; Sotoudeh and Mardani, 2018). Moreover, the presence of complex polysaccharides in red seaweeds and resistance to enzymatic degradation in the stomach and small intestine (Zheng et al., 2020) are the reasons for the negative effects observed on the intestinal proteolytic activity of gilthead sea bream, *Sparus aurata* fed *Gracilaria sp* (Vizcaíno et al., 2016). Thus, reduced growth and nutrient utilization (low bioavailability from the alga) and alga consumption-induced gut morphological alterations (reduce intestinal absorptive capacity) are associated with *Gracilaria* sp. feeding. Hence innovative strategies to increase the efficacy of *Gracilaria* sp. in improving the growth and health of farmed fish must be examined thoroughly before the alga could become an added-value sustainable ingredient for aquafeeds.

Cell wall disruption methods should be tested as ways to improve the bioavailability of algal nutrients. Some cell wall disruption techniques are known to increase nutrient accessibility

and digestibility, by breaking the cell wall whilst maintaining nutritive quality (Teuling et al., 2019). Furthermore, physical grinding is recommended for better extraction of protein from the macroalgae *Porphyra acanthophora*, *Sargassum vulgare* and *Ulva fasciata* (Barbarino and Lourenço, 2005). Moreover, physical-mechanical processing of *Gracilaria gracilis* augmented the content of low-molecular weight protein and peptides, and the processed alga increased the dry matter (19%), protein (4%) and energy (22%) apparent digestibility coefficients (ADCs) in European sea bass *Dicentrarchus labrax* compared to the fish fed unprocessed algae (Batista et al., 2020a).

Another strategy to optimize the nutrient utilization and gut health is dietary supplementation of functional additives that support animals' health and stress resistance (Encarnação, 2016). In this regard, phytogenics, algal polysaccharides and nucleotides are some additives that are preferred by the aquaculture sector. Phytogenics are plant-derived natural bioactive compounds that have been tested in a number of species, and are reported to have positive effects on nutrient digestibility, animal growth and health (Applegate et al., 2010; Yang et al., 2015). The use of such natural substances are gaining interest within the aquaculture industry, mainly for improving animal performance, immune response and disease resistance (Encarnação, 2016). Among these additives, essential oils like carvacrol and thymol from oregano (*Origanum vulgare*) have been shown to improve feed conversion (FCR) and antioxidant-related protective capacities in rainbow trout (Giannenas et al., 2012), channel catfish *Ictalurus punctatus* (Zheng et al., 2009) and European sea bass (Volpatti et al., 2013). In gilthead sea bream fed essential oils, improved absorptive capacity of the intestine and improved feed gain ratio were linked to anti-inflammatory and anti-proliferative intestinal transcriptomic profile (Pérez-Sánchez et al., 2015). Likewise, dietary limonene enhanced growth in Nile tilapia through the regulation of genes involved in nutrient absorption and transport, lipid assimilation and antioxidant enzyme defence (Aanyu et al., 2018).

As for the algal polysaccharides, alginate, one of the most widely produced algal polysaccharides, is naturally present in brown seaweeds cell walls and is composed of β-D mannuronic acid and α-L guluronic acid monomers. Alginate oligosaccharide (AOS), depolymerised from alginate, has several biological properties and has received much attention due to its beneficial effects. This additive has the ability to not only improve intestinal morphology (e.g. intestinal villus height and goblet cell counts) and barrier function, but also shape microbiota and enhance both growth and health of animals (Wan et al., 2018; Wang et al., 2006; Yan et al., 2011). In Atlantic salmon *Salmo salar* the dietary inclusion of AOS induced a potential prebiotic effect on microbiota, favoring certain beneficial gut

140 microorganisms with carbohydrate-active enzymes (Gupta et al., 2019). Sodium alginate and
141 alginic acid extracted from *Laminaria* sp. were also shown to enhance innate immunity,
142 disease resistance, feed utilization and growth of several fish species (Ashouri et al., 2020;
143 Harikrishnan et al., 2011; Van Doan et al., 2017; 2016) including European seabass (Bagni et
144 al., 2005).
145 Dietary nucleotides are a group of additives that are widely used in aquaculture as feed
146 attractants. They are often implicated in numerous positive physiological effects including
147 increased growth performance, feed utilization, and enhanced intestinal fold morphology in
148 several fish and shellfish species (Burrells et al., 2001; Hossain et al., 2019; Safari et al.,
149 2015). In addition, nucleotides were shown to have a protective effect in overcoming
150 intestinal and inflammatory reactions induced by plant-rich protein diets (de Rodrigáñez et al.,
151 2013), could boost the immune response in hybrid striped bass, *Morone chrysops* x *Morone*
152 *saxatilis*, carp, *Cyprinus carpio* and crayfish, *Astacus leptodactylus* (de Cruz et al., 2020;
153 Safari et al., 2015; Sakai et al., 2001) and could attenuate stressor-induced plasma levels of
154 cortisol in sole, *Solea solea* (Palermo et al., 2013). To understand the efficacy of the
155 abovementioned feed additives and processing techniques, feeding experiments should be
156 conducted to assess the nutrient bioavailability, growth and health of fish fed *G. gracilis*.
157 As novel approaches, here we assessed the effectiveness of a physical-mechanical rupture
158 method and the inclusion of feed additives (phytogenic compounds, alginate oligosaccharide
159 and nucleotides) as strategies to enhance the utilization of *G. gracilis* in diets for European
160 seabass. The nutrient utilization (specific growth ratio, feed conversion ratio, apparent
161 digestibility coefficients, nutrient balance, intestinal brush border membrane enzyme
162 activities and plasma metabolic parameters), gut integrity, antioxidant and immunological
163 status of fish were evaluated after feeding the fish for 106 days.
164

2. Materials and methods

This study was carried out by accredited scientists in compliance with European Union (directive 2010/63/EU) and Portuguese (Decreto-Lei nº 113/2013, de 7 de Agosto) guidelines on the protection of animals used for scientific purposes. The experiment was approved by CIIMAR animal welfare body (ORBEA-CIIMAR) and by the national competent authority (Direção Geral de Alimentação e Veterinária - DGAV).

2.1. *E*xperimental diets

Six isoproteic (53.5 % dry matter, DM) and isoenergetic (22 kJ kg DM) diets were formulated and extruded by SPAROS Lda. (Olhão, Portugal), based on the known nutritional requirements of European seabass (NRC, 2011). A commercial feed-like control diet (CTRL) was compared with five experimental diets containing 8.3% of *G. gracilis*. This seaweed was commercially produced under an IMTA system (ALGAplus, Ílhavo, Portugal) and we tested both unprocessed (diet GRA) and processed product, i.e. after physical processing (diet GRAP). The present trial was part of a larger experiment where different unprocessed algae (*G. gracilis and Nannochloropsis oceanica)* were compared to a control (CTRL) diet in European sea bass; the CTRL and GRA diets are the same in this and our previous study (Batista et al., 2020b). A physical-mechanical rupture method (Patent WO/2019/171293; Valente et al., 2019) was applied to this algae using a vibratory grinding mill (Siebtechnik TS250, Geldern, Germany). The resulting algae biomass was entirely dried by convection, at 50 ºC, in a pilot-scale tray dryer (Armfield UOP8, Ringwood, England) prior its use as feed ingredient. Three other diets were formulated by supplementing the GRA diet with either 0.02% phytogenic compounds (Digestarom P.E.P. MGE 150, Biomin GmbH, Herzogenburg, Austria; diet PHY), or 2.5% of alginate oligosaccharide (Centre d'Etude et de Valorisation des Algues (CEVA), Pleubian, France; diet AOS) or 0.08% free nucleotides (NUCLEOFORCE FISH™, BIOIBERICA, Spain; diet NUC), according to the product recommendation levels. Digestarom P.E.P. MGE 150 is a blend of encapsulated phytogenic compounds that contain anise, citrus, and oregano essential oils; their main active compounds are anethol, carvacrol, thymol and limonene (Peterson et al., 2014). The alginate oligosaccharide is a prebiotic candidate derived from the macroalga *Laminaria* sp. by CEVA. Briefly, commercial-grade sodium alginate, Satialgine S 60 NS (Cargill, France) was depolymerized to produce the oligomeric form of sodium alginate. Depolymerization was performed using an enzymatic process based on bacterial alginate-lyase, as described in the

patent EP0979301. B1NUCLEOFORCE FISH™ is a concentration of free nucleotides and active precursors obtained from yeast.

The formulation and proximate composition of the experimental diets are provided in Table 1. For the determination of the apparent digestibility coefficients (ADCs), 1% chromium oxide (Cr_2O_3, Merck KGaA, Germany) was added as inert marker to each experimental diet. Extruded diets were ground and mixed with the marker and dry pelleted through a 3.2 mm die at 50 °C using a laboratory pellet press (CPM, C-300 model, S. Francisco, USA).

2.2. Growth trial

European seabass juveniles were transported to CIIMAR (Matosinhos, Portugal) from a fish farm (SONRIONANSA S.L., Cantabria, Spain) and kept in quarantine for 2 weeks to adapt to the new rearing facility and environmental conditions (water temperature of 21 °C, salinity of 35‰, flow rate at 4 $Lmin^{-1}$ and 12 h light/12 h dark photoperiod regime). Fish were fed with a commercial diet (AQUASOJA – 49% crude protein, 20% crude fat). After acclimation, fasted fish (24 h period) were anesthetized (75 mg L^{-1} of MS222; Sigma-Aldrich Co. LLC, Bellefonte, USA) and individually weighed (g) to establish eighteen homogeneous groups of nineteen fish each (average body weigh of 29.7 ± 0.02 g; initial density of 11.3 kg m^{-3}) for each study group. The fish were distributed into 50 L fiberglass tanks that were part of a saltwater recirculation system (water temperature of 21 °C, salinity of 35‰, flow rate at 4 $Lmin^{-1}$ and 12 h light/12 h dark photoperiod regime). Each diet was tested in triplicate and fish were fed each diet close to apparent satiation, three times a day, by automatic feeders, for 106 days. The amount of feed supplied to each tank was daily adjusted based on the presence or absence of uneaten feed remaining in the bottom of the tank after each meal. When all feed distributed to a tank by the automatic feeders was quickly ingested, in the following day, the daily total amount of feed distributed to that tank was augmented by 5%. When some uneaten pellets remained in the bottom of a tank, the daily dose was reduced by 5 %, until no feed losses were recorded. Any non ingested feed pellets were collected after each meal and weighed for determination of daily feed intake. Nitrogenous compounds (ammonia and nitrite nitrogen; <0.4 mgL^{-1}) and pH (7.7) were monitored during the trial and kept at levels recommended for marine species (Kır et al., 2019; Weirich and Riche, 2006).

Ten fish from the initial fish stock, and five fish per tank at the end of the trial, were sacrificed by employing an anesthetic overdose (150 mg L^{-1} of MS222) after a 24 h fasting period. These fish were frozen at -20 °C for further whole body composition analysis. At the end of the growth trial, and for the determination of brush border membrane (BBM) enzyme

activity, four fish per tank were anesthetized with 75 mg L^{-1} of MS222, after a 5 h fasting period, individually weighed (g) and sacrificed with a sharp blow on the head. The pyloric caeca (PC), anterior intestine (AI, section further down the PC until the start of the posterior intestine indicated by increased diameter) and posterior intestine (PI, the terminal part of the intestine with larger diameter, until the anus) were sampled and kept at -80 °C until further analysis. After a 24 h fasting period, all the remaining fish were anesthetized (75 mg L^{-1} MS222), and individually weighed (g). Blood was collected from the caudal vein of four fish per tank. Plasma was obtained after centrifuging the blood at 5000x*g*, for 10 min at 4 °C, and stored at -80 °C for analysis of metabolite levels and innate immune parameters. Fish were then sacrificed by a sharp blow on the head to collect and register viscera and liver weights. Livers were immediately frozen in liquid nitrogen and kept at -80 °C until determination of oxidative stress parameters. A sample of the left dorsal muscle (≈5 g) was collected for the determination of its chemical composition. A 0.5 cm cross-section from the anterior (after the pyloric caeca) and posterior (before the rectum sphincter) intestine was washed and fixed in 10% neutral-buffered formalin for 24 h, preserved in ethanol 70% until being processed according to standard histological procedures.

2.3. Digestibility trial

After the growth trial, the remaining fish in each tank were transferred to a system with similar tanks, but specially designed for digestibility studies (Guelph system), as suggested by Cho and Slinger (1979) to evaluate the ADC of the experimental diets. Fish were subjected to the same rearing conditions (water temperature, salinity, nitrogenous compounds, flow rate and photoperiod regime) as described for the growth trial, and they were fed twice a day to apparent satiation the experimental diets with Cr_2O_3 as inert marker. After a 10 days' adaptation period to these diets, feces were daily collected during 4 weeks, before feeding (9:00 and 16:00), centrifuged (5100x*g*, 5 min, 4°C) and frozen at -20 °C. After each meal, tanks were carefully cleaned to remove all uneaten feeds from the bottom of the tanks and the sedimentation column.

2.4. Proximate analysis

Whole fish collected from each tank were ground and pooled to determine the moisture content (105 °C for 24 h). The homogenized carcasses, dorsal muscle and feces were freeze-dried before further analysis. All chemical analyses, including diets, tissues and feces, were performed in duplicates, by following the methods of AOAC (2006). The proximate

composition of the samples were analyzed: DM after 24 h at 105 ºC; ash after combustion at 500 ºC for 5 h in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany;); crude protein (N × 6.25) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA); fat content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany); gross energy by an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany); and total phosphorus of digested ash by spectrophotometry at 820 nm using ammonium molybdate according to ISO 13730:1996 (1996). Chromic oxide content in diets and feces was determined according to the method of Bolin et al. (1952). The crude fiber content in the alga and feeds was analyzed as neutral detergent fiber (NDF) according to ISO 16472:2006 (Robertson and Van Soest, 1981; Van Soest and Robertson, 1985).

2.5. Intestine histomorphology

The two most representative fish per tank, in terms of body weight, were selected for the histology (6 fish per diet) study. Briefly, cross-sections (3 µm) from the anterior and posterior intestine were obtained using a semi-automated rotary microtome (Leica RM 2245). The obtained sections were then stained with Alcian Blue/PAS (pH 2.5) and examined under a light microscope (Olympus BX51, GmbH, Hamburg, Germany) with a camera (Olympus DP50). An imaging software (Olympus cellSens Dimension Desktop) was used to measure cross sectional area (mm^2), *villus* length and width (µm), *muscularis* externa (µm), and neutral and acid goblet cells (nº GC per fold), in two sections of each anterior and posterior intestinal sample, as previously detailed by Batista et al. (2020b).

2.6. Intestinal brush border membrane (BBM) enzyme activities

Intestinal sections (pyloric caeca, anterior and posterior intestine) were gently squeezed out to remove the remaining content. Tissue samples were then diluted 1:10 (w:v) in iced saline buffer and crushed using a tissue-lyser disruption system (Tissue Lyser II, Qiagen, Germany) at 30Hz for 1min. Samples were centrifuged at 13.500x*g* for 10 min at 4°C and the supernatant was used to measure the BBM enzyme activities (Messina et al., 2019). The hydrolysis of maltose and sucrose, by the BBM enzyme maltase and the complex sucrase-isomaltase (SI), was determined according to Harpaz and Uni (1999). Intestinal alkaline phosphatase (IAP) and γ-glutamyl transpeptidase (γ-GT) activities were determined using commercial kits (Paramedical, Pontecagnano Faiano, SA, Italy), following the instructions of the manufacturer. One unit (U) of enzyme activity corresponded to the amount of enzyme that

transforms or hydrolyses 1 μmol of substrate mL^{-1} min^{-1}. The specific enzyme activity was calculated as U of enzyme activity *per* g of tissue.

2.7. Plasma metabolic parameters

Commercially available kits (Biochemical Enterprise, Milan, Italy) were used to determine plasma parameters using an automated analyser system for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy) The following parameters were determined according to the manufacturer's protocols: glucose (Glu, mg dL^{-1}), cholesterol (Chol, mg dL^{-1}), triglycerides (Trig, mgd L^{-1}), total proteins (TP, gd L^{-1}) and albumin (Alb, g dL^{-1}).

2.8. Oxidative stress analysis

Liver samples were homogenized using phosphate buffer (0.1 M, pH 7.4) in a proportion of 1:10 (w:v). The protein content was determined according to the procedures of Bradford (1976) and the values were used to standardize antioxidant enzymes activities. The following parameters were measured in triplicates using a microplate reader. Concentration of total antioxidant in samples was determined by using the total antioxidant capacity assay kit (Sigma MAK187), by measuring the formation of Trolox equivalents. Total glutathione (TG) was evaluated at 412 nm after the formation of 5-thio-2-nitrobenzoic acid (TNB), as detailed in Baker et al. (1990). Formation of TNB was monitored by spectrophotometry at 415 nm, for 7 min, with results expressed as nmol TNB conjugated formed per min per mg of protein. Glutathione peroxidase (GPx) was evaluated based on NADPH oxidation at 340 nm (Mohandas et al., 1984) through an indirect method based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx. The reaction was performed at 25 ºC and pH 8.0, using H_2O_2 and including sodium azide (NaN_3) as a catalase inhibitor. Oxidation of NADPH was recorded spectrophotometrically at 340 nm at 25 ºC, after which the enzyme activity was calculated as nmol NADPH oxidised/min/mg of protein. Glutathione s-transferase (GST) was determined as described by Habig et al. (1974). Total activity (cytosolic and microsomal) was determined by measuring the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). The change in absorbance was recorded at 340 nm and 25 ºC for 5 min and enzyme activity was calculated as mmol CDNB conjugate formed per min per mg of protein. Catalase (CAT) activity was measured based on a study by Claiborne (1985), using hydrogen peroxide (H_2O_2) 30% as substrate. Changes in absorbance were recorded at 240 nm at 25º C. CAT activity was calculated in terms of mmol H_2O_2 consumed per min per mg of protein. Lipid peroxidation (LPO) was determined

according to Bird and Draper (1984), by quantifying the presence of thiobarbituric acid reactive substances (TBARS), namely malondialdehyde (MDA). The decomposition of unstable peroxides derived from polyunsaturated fatty acids (PUFAs) induces the formation of MDA, which was quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBA). The absorbance of each aliquot was measured at 535nm and the rate of LPO was expressed as nmol of MDA formed per gram of fresh tissue.

2.9. Innate immune parameters analysis

Lysozyme activity (EU min^{-1} mL^{-1} plasma) was determined based on the microtitre method described by Hutchinson and Manning (1996) adapted from Ellis (1990). One lysozyme enzyme unit (EU) was defined as the amount of lysozyme that caused a decrease in 1 OD absorbance *per* min. Total peroxidase activity (EU mL^{-1} plasma) was measured following the procedure described by Quade and Roth (1997) and Costas et al. (2011), and was determined by defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) was based on the lysis of rabbit red blood cells (2.8×10^8 cells mL^{-1}; Probiológica, Belas, Portugal) that were used as target cells in the presence of ethylene glycol tetraacetic acid (EGTA; Sigma) and Mg^{2+} ($MgCl_2 \cdot 6H_2O$; VWR) as described by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma that causes 50 % lysis of cells.

2.10. Calculations

ADCs of the experimental diets were calculated according to Maynard et al. (1979): DM ADC (%) = 100 × (1 – (dietary Cr_2O_3 level/feces Cr_2O_3 level)) and nutrients ADC (%) = 100 × (1 – (dietary Cr_2O_3 level/feces Cr_2O_3 level) × (feces nutrient or energy level/dietary nutrient or energy)); Specific growth rate = 100 × Ln (Final body weight) – Ln (Initial body weight))/days; Average body weight (ABW) = (final body weight + initial body weight)/2; Voluntary feed intake (%)= 100 × crude feed intake/ABW/day; Feed conversion ratio = dry feed intake/weight gain; Hepatosomatic index = 100 × liver weight/body weight; Viscerosomatic index = 100 × weight of viscera/body weight; Digestible nutrient or energy (E) intake = (dry feed consumption × Nutrient (%) or E (kJ/g) in the diet × ADC Nutrient or E/ABW/days); Nutrient or E gain= (final carcass nutrient or E content – initial carcass nutrient or E content)/ABW/days; Nutrient or E retention efficiency (% Digestible nutrient or E) = (Nutrient or E gain/Digestible nutrient or E intake) ×100; Fecal nutrient or E losses (mg/100g ABW/day) = Crude nutrient intake (mg/100 g ABW/day) x (1 – (ADC Nutrient or

E/100)); Non-fecal nutrient losses (mg/100 g ABW/day) = Digestible nutrient intake (mg/100 g ABW/day) – nutrient gain (mg/100 g ABW/day); Non-fecal E losses (mg/100 g ABW/day) = Non-fecal N losses x 24.9 kJ/g N; Metabolizable energy (kJ/kg ABW/day) = digestible E intake (kJ/kg ABW/day) – Non-fecal E losses (kJ/ kg ABW/day); Total heat production (kJ/kg ABW/day) = E gain (kJ/kg ABW/day) - Metabolizable E (kJ/kg ABW/day).

2.11. Statistical analysis

Data were analyzed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Whenever necessary dependent variables were transformed to adhere to the assumptions of the selected statistical test. Data were analyzed using a one-way ANOVA with the statistical program IBM SPSS STATISTICS, 25.0 package, IBM corporation, New York, USA (2017). When the output was significant, individual means were compared using HSD Tukey Test. When data did not meet the assumptions of ANOVA, Kruskal Wallis test was performed followed by the Dunn test with Bonferroni correction to identify significant differences between groups. In all cases, the minimum level of significance was set at $P < 0.05$. Principal component analysis (PCA) was performed to assess the variables that differed significantly among dietary treatments using XL-STAT 2020® system software (Addinsoft, USA).

3. Results

Growth performance and nutrient utilization

The dietary inclusion of *G. gracilis* did not have a significant effect (P>0.05) on fish growth performance (Table 2). The dietary inclusion of additives or technological processing was not effective in increasing the fish final body weight significantly, in spite of the apparently higher weight gain observed in all the experimental groups. The feed intake and the feed conversion ratio were also not significantly affected by the dietary treatments.

The hepatosomatic index (HSI) was affected by the dietary inclusion of *G. gracilis*; fish fed either GRA, or PHY or AOS or NUC diets had significantly lower indexes (1.7-1.8) than those fed the CTRL diet (2.1). Significant differences were not detected for the final whole body or muscle composition of fish fed the different diets (Table 2), although fish fed the CTRL had apparently higher muscle fat content.

The experimental diets impacted the apparent digestibility coefficient (ADC) of nutrients and energy significantly (P<0.05, Table 3). The dietary inclusion of *G. gracilis* in GRA diet resulted in a significant decrease in dry matter ADC (16%) and nutrient ADCs compared to the CTRL, but processing of *G. gracilis* (GRAP) yielded ADCs values similar to that of the CTRL diet. All the tested additives significantly lowered the dry matter ADC compared to the CTRL, but in fish fed NUC diet (31%) values did not differ significantly from those observed in GRAP. Likewise, protein ADC value was significantly reduced in GRA diet (68%) compared to the CTRL, resulting in the highest fecal and lowest non-fecal N losses. Technological processing (as observed in GRAP, 84%), and additives AOS (77%) and NUC (78%) did not alter the protein ADCs compared to the CTRL diet (85%). The digestible N intake was significantly reduced in fish fed GRA compared to all other treatments. Furthermore, both the processing of the algae, and the use of additives increased digestible N ingestion, resulting in a significantly higher N gain in fish fed GRAP, PHY or AOS compared to CTRL. The dietary inclusion of *G. gracilis* resulted in the highest N retention efficiency (24-29% vs 22% in the CTRL diet), but only fish fed GRA differed significantly from the CTRL. Lipid ADC in the GRAP fed fish were higher compared to those fed GRA diet.

Energy ADC was significantly lower in fish fed GRA (38%), PHY (43%) and AOS (48%) compared to the CTRL (64%) (Table 3). But energy ADCs in fish fed GRAP and NUC diets (64% and 53%, respectively) were similar to CTRL (64%), leading also to similar energy balance in these three diets. Metabolizable energy increased significantly in fish fed GRAP in relation to those fed GRA. Moreover, fish fed both AOS and NUC diets approached values

comparable to those observed in fish fed the CTRL diet. Energy gain remained similar among fish, but energy retention efficiency was highest in fish fed GRA, PHY and AOS diets (45-56%).

Phosphorus digestibility was not significantly affected by the dietary treatments (51-59%) resulting in similar P balance (Table 3).

Intestinal morphology

Intestinal morphology was generally very well preserved in all fish but diets had a significant impact on the anterior intestine (Fig. 1; Table 4). Fish fed GRAP had significantly larger *villi* width (151 µm) than those fed GRA (113 µm) and PHY (111 µm). Diet GRAP also induced the formation of more acid GCs, but without differing significantly from fish fed the CTRL, GRA, PHY and NUC diets. The dietary supplementation with alginate oligosaccharide (AOS diet) resulted in a significant reduction in the number of acid GC in the anterior intestine, compared to fish fed GRA, GRAP or PHY diets. The differences observed in the anterior intestine were not be observed in the posterior intestine.

Intestinal BBM enzyme activities

The specific activities of intestinal maltase, sucrase-isomaltase, alkaline phosphatase and γ-glutamyltransferase are presented in table 5. In fish fed AOS, the specific activity of maltase in the pyloric caeca was the lowest, being significantly different from the values observed in CTRL and NUC groups (2138.5 < 2770.2 < 2936.5 µm min^{-1} g^{-1} respectively; $P<0.05$). The NUC group had the highest SI value (1119.6 µm min^{-1} g^{-1}), which was not different from that of the control group, but was higher than those of the other treatment groups ($P<0.05$). A significant difference ($P<0.05$) in the IAP specific activity was noticed only between AOS (88.0 µm min^{-1} g^{-1}) and GRAP (62.9 µm min^{-1} g^{-1}) groups, and these groups had the highest and the lowest values. The additives or processing did not significantly affect the γ-GT specific activity

In the anterior intestine the inclusion of Digestarom (PHY) significantly decreased the activity of maltase compared to the control and AOS groups; 4211.8 < 5793.5 < 6082.2 µm min^{-1} g^{-1}; $P=0.001$. Fish fed GRA diet exhibited a lower SI specific activity compared to the control group (1499.0 *vs* 2238.5 µm min^{-1} g^{-1}, $P<0.05$). The dietary supplementation of alginate (AOS) significantly increased the IAP activity in the anterior intestine compared to all the other groups except the CTRL (174.4 *vs* 104.7-120.6 µm min^{-1} g^{-1} in *Gracilaria*-rich diets).

Maltase activity was also significantly higher in AOS compared to GRA. No significant differences were observed for the γ-GT values.

In the posterior intestine no significant differences could be noticed in maltase specific activity between treated and control groups. The supplementation of alginate (AOS) or the physical treatment of *G. gracilis* biomass (GRAP) triggered the maltase activity compared to diets supplemented with Digestarom (PHY) or nucleotides (NUC) (5509.3-5695.3 *vs* 3606.5-3972.1 µm min^{-1} g^{-1}, $P<0.05$). Although we did not observe any significant differences in the SI and IAP specific activities, the physical treatment of *G. gracilis* (GRAP) strongly affected the specific activity of γ-GT (14.3 µm min^{-1} g^{-1}; $P<0.001$) compared to the other treatments.

Plasma metabolic parameters

Plasma glucose and total protein levels were not significantly affected by the dietary treatments. Their values ranged from 120.9 to 149.6 mg dL^{-1} and 3.9 to 4.2 g dL^{-1} respectively (Table 6).

The supplementation of nucleotides (NUC) significantly decreased total cholesterol levels compared to the CTRL and GRA diets (140.6 vs 169.4-187.3 mg dL^{-1}, $P<0.05$). The control group had the highest value of plasma triglycerides (436.8 mg dL^{-1}). The inclusion of *G. gracilis* significantly decreased the triglycerides, and the value of CTRL (436.8) was significantly different from those of GRA, GRAP, PHY and AOS groups (316.2 > 298.5 > 273.7 > 237.7 mg dL^{-1}, respectively).

The plasma albumin levels in the control and dietary groups did not differ significantly. The supplementation of alginate or nucleotides significantly decreased the albumin level in fish fed diets with *G. gracilis* (0.85 vs 1.10 mg dL^{-1}, $P<0.05$).

Oxidative stress

The dietary inclusion of *G. gracilis* led to a general increase in LPO with significantly higher values in fish fed PHY and AOS (58.1 and 57.6 nanomol TBARS g $tissue^{-1}$, respectively) compared to fish fed the CTRL diet (48.4 nanomol TBARS g $tissue^{-1}$) (Fig 2). The dietary treatments did not affect TG, GST and GR liver contents significantly. CAT activity was lowest in fish fed PHY (34.5 µmol min^{-1} mg $protein^{-1}$), which was significantly different from values in the liver of fish fed the CTRL diet (66.0 µmol min^{-1} mg $protein^{-1}$). GPx was lowest in fish fed NUC (2.1 µmol min^{-1} mg $protein^{-1}$) compared to those fed GRAP (6.2 µmol min^{-1} mg $protein^{-1}$).

Humoral non-specific immune parameters

The humoral non-specific immune parameters evaluated in plasma did not show any differences between treatments, with lysozyme values ranging from 824 to 975 EU mL^{-1}; the peroxidase between 182 and 324 EU mL^{-1} and complement activity between 154 and 239 Units mL^{-1} (Table 6).

Principal component analysis (PCA)

The PCA analysis helps to differentiate the samples based on the measured variables, and it enables to explain the pattern of interrelationships among a larger set of variables. According to the biplot in Figure 3a, the first two components (F1 and F2) of the plot explain 62.2% of the variability of the experimental data. Fish fed the CTRL and GRAP diets are clearly separated from GRA, PHY, NUC and AOS along F1 with 36.1% of the variance. A second projection is proposed in Figure 3b, just including variables related to nutrient utilization (nutrient digestibility, intestine morphology and enzyme activity in intestine). Based on this biplot, F1 and F2 captured 62.7% of the variability of the experimental data. Fish fed GRAP, CTRL and AOS are separated from those fed GRA, PHY and NUC along F1 with 36.8% of the variance.

Discussion

The capacity of seaweeds to be natural sources of nutrients and bioactive compounds in diets for different fish species has been assessed in several studies. In European sea bass, *Gracilaria* species have been used as sustainable dietary ingredients to replace fish meal (Batista et al., 2020b; Valente et al., 2006), but results evidenced poor nutrient utilization and reduced digestibility that allow only moderate levels of inclusion of the alga in aquafeeds. Algae cell walls are composed of structurally complex and heterogeneous polysaccharides that may limit accessibility to algal proteins (e.g. lectins and phycobiliproteins) (Fleurence et al., 1995; Harnedy and FitzGerald, 2011), affect intestinal morphology and the digestive processes in fish (Granby et al., 2020; Sotoudeh and Mardani, 2018). Moreover, the major non-fibrillar polysaccharides extracted from *Gracilaria* sp. are soluble sulphated galactans (agar) (Rodríguez et al., 2009) that may also resist enzymatic degradation in the stomach and small intestine Zheng et al. (2020). In our earlier study we tested the effect of 8% *G. gracilis* in diets for European sea bass (Batista et al., 2020b) and reported that alga feeding impaired protein and energy ADC values, based on the comparison of CTRL and GRA diets. This result was attributed to a limited access of the digestive enzymes to algal proteins, which prompted us to further evaluate the potential of processing technologies or feed additives to mitigate the negative effects caused by GRA diet. In the present study, the ability of fish to digest seaweed-rich diets was largely improved by the processing of *G. gracilis,* without affecting fish growth in a significant way. The protein and energy ADCs in the fish fed processed *G. gracilis* (GRAP) were significantly higher than values observed in fish fed the unprocessed algae (GRA) and similar to the CTRL diet. These results, together with the significantly higher γ-GT activity (posterior intestine) observed in the fish fed GRAP compared to those fed the unprocessed one (GRA) or CTRL diet could be indicating the efficacy of the processing technology. The activity of γ-GT, which contributes to the final protein digestion on the microvilli surface, was highest in the posterior intestine as previously reported in earlier studies (Messina et al., 2019). Although Messina et al. (2019) did not find an effect of alga on this enzyme, our processing technique influenced it. This very positive effect of the physical process in improving the *G. gracilis* nutrient utilization was a major achievement that could not be attained by any of the tested additives. These results reinforce previous observations on increased nutrient accessibility and bioavailability from physical-mechanical processed *G. gracilis* compared to unprocessed algae (Batista et al., 2020a). According to Tulli et al. (2017), cell wall disruption technologies can release proteins, lipids and other naturally hydrophobic components and increase their digestion and nutrient

absorption rate by fish. Nutrient absorption could be further enhanced by the significantly larger *villus* width, as observed in fish fed the GRAP diet compared to those fed GRA. Previous studies associated a significant reduction of intestinal absorption area and *villi* length or width with growth impairment and lower nutrient uptake in fish fed seaweed-rich diets (Araújo et al., 2016; Moutinho et al., 2018; Silva et al., 2015).

The use of feed additives in *Gracilaria*-rich diets was less efficacious in improving European sea bass nutrient and energy ADCs, but have still improved the overall digestibility of those diets. Fish fed alginate oligosaccharide had apparently or significantly higher IAP activity in pyloric caeca and intestinal segments compared to all other *Gracilaria*-rich diets, that resulted in a protein ADC that did not differ from the CTRL diet. It should be noted that fish fed AOS had the lowest number of acid caliciform cells in the anterior intestine indicating a possible effect on the barrier function. According to Deplancke and Gaskins (2001), acid caliciform cells confer protection against bacterial translocation. In Atlantic salmon, the supplementation of diets with this same alginate oligosaccharide caused an overall reduction in bacterial diversity of the distal intestine bacterial community compared to the control fish (Gupta et al., 2019). The impact of the experimental diets on intestinal microflora was not evaluated in European sea bass but merits further evaluation. As regards the nucleotide, it resulted in protein and energy ADC values similar to those observed in the CTRL diet. The nucleotide diet also triggered the highest activity of both maltase and SI in pyloric caeca that could be partially contributed to the higher energy ADC value of this group. Moreover, both the nucleotides and the alginate oligosaccharide seem to have counteracted the decreased intestinal *villus* width observed in fish fed GRA. This is in agreement with previous findings reporting a protective role of nucleotides against intestinal and inflammatory reactions induced by the consumption of a diet containing a high amount of vegetable ingredients (de Rodrigáñez et al., 2013). The dietary supplementation of phytogenics did not improve nutrient digestibility in a significant way, compared to the GRA diet, resulting in higher fecal N and energy losses compared to the CTRL diet. Phytogenic diet also significantly lowered the maltase activity in the anterior intestine compared to the CTRL. Although previous studies reported improved absorptive capacity of the intestine of gilthead sea bream fed essential oils (Pérez-Sánchez et al., 2015), this could not be confirmed in the present study as fish fed PHY had the lowest *villus* length.

The present study results highlight the metabolic capacity induced by the dietary inclusion of *Gracilaria* on European sea bass. It is worth noticing the significant reduction of plasma cholesterol in fish fed NUC and a reduction in triglycerides in fish fed GRAP, PHY and AOS

diets. There was a parallel reduction of HSI in those fish, similar to a previous report on gilthead sea bream fed *Gracilaria* sp. (Vizcaíno et al., 2016). In rainbow trout, the dietary inclusion of seaweeds significantly affected lipid metabolism; a downregulation of *fas* (involved in de *novo* fatty acid biosynthesis pathways) in fish fed 4% *S. latissima* together with an increasing trend for *cpt1b1* expression (carnitine palmitoyltransferase regulates the long-chain fatty acid beta-oxidation), were associated with a significant reduction of HSI in those fish (Ferreira et al., 2020). In red sea bream *Pagrus major* fed *Spirulina* sp., reduced total lipids both in serum and liver were also associated with elevated activity of carnitine palmitoyltransferase (Nakagawa et al., 2000). Nevertheless, in the present study, the experimental diet did not significantly affect the whole body or muscle fat content. However, lipid ADC in fish fed the processed alga was apparently or significantly higher compared to those fed the CTRL or the unprocessed alga. A longer feeding trial would probably be needed to clearly understand the lipid-lowering effects of *Gracilaria*-rich diets.

Humoral immune parameters evaluated in our study (peroxidase, lysozyme and ACH50) were not affected by the dietary inclusion of *Gracilaria* sp., although previous studies often report contradictory results. The immunological response was enhanced in rainbow trout (Araújo et al., 2016) whilst ACH50 was significantly decreased in European sea bass fed *Gracilaria* sp. (Peixoto et al., 2016a). The supplementation of fish diets with phytogenic compounds has been implicated in enhancement of immune competence through complement system activation and serum lysozyme levels stimulation in several fish species (Abo-State et al., 2017; Diler et al., 2017; Peterson et al., 2014; Yang et al., 2015). On the other hand, oligo-alginate supplementation has been shown to increase the ACH50 and lysozyme activities of European seabass (Bagni et al., 2005). Although such effects could not be presently observed, we cannot disclose the effectiveness of the alga in promoting immune competence of fish under an environmental stress scenario.

Gracilaria is a genus of red seaweed rich in functional polysaccharides and antioxidant compounds (Holdt and Kraan, 2011). These exogenous antioxidants may exert a protective role against oxidative stress in the liver of fish (Magnoni et al., 2017; Peixoto et al., 2016a; 2016b). However, in the present study the inclusion of 8% of *Gracilaria* sp. (GRA and GRAP diets) had no significant impact on fish liver antioxidant defence system compared to the CTRL diet. Phytogenic compounds (Aanyu et al., 2018), oligosaccharides (Özlüer-Hunt et al., 2011; Torrecillas et al., 2012; Torrecillas et al., 2013) and nucleotides (Hossain et al., 2016) have all been recognized as beneficial for fish antioxidant defence systems, albeit always such an effect is dependent on inclusion levels. In the present study, the dietary inclusion of either

0.02% of phytogenic compounds (PHY diet) or 2.5% alginate oligosaccharide (AOS diet), did significantly increase the lipid peroxidation compared to the CTRL diet. A concomitant decrease in catalase (CAT) activity, might have contributed to increased lipid peroxidation, especially in fish fed PHY. On the other hand, the inclusion of 2.5% nucleotides lowered the glutathione peroxidase (GPx) activity. In this case, no major impact was observed in LPO levels, suggesting that dietary nucleotides provide exogenous antioxidants that lessen the need for endogenous production of antioxidant enzymes. These results suggest a limited effect of the selected feed additives on European sea bass oxidative stress at the tested doses.

According to the first PCA plot, diets projected positively along F1 (CTRL and GRAP) have a strong correlation with variables such as protein and energy ADCs, the respective fecal losses, villus width and γ-GT activity in the anterior intestine, and with muscle lipids. These results highlight the ability of the algae technological processing to counteract the negative effects observed in fish fed GRA. Moreover, figure 3b unveils the effects of dietary additives, evidencing the proximity of AOS to the CTRL mainly due the activity of anterior intestine enzymes and protein and energy ADCs.

Conclusion:

The ability of fish to digest seaweed-rich diets was largely improved by the technological processing of *G. gracilis*, although lacking an effect on fish growth. This major achievement was associated with increased digestibility of protein and energy, resulting in ADC values similar to the CTRL. The use of feed additives in *Gracilaria*-rich diets was less efficacious in improving European sea bass nutrient and energy ADCs, but have still improved the overall digestibility of those diets. Fish fed alginate oligosaccharide was mainly associated with increased activity of anterior intestine enzymes. Moreover, the algae technological processing and both the nucleotides and the alginate oligosaccharide were able to maintain the intestinal villus width rather than decrease it as in the case of fish fed GRA. The tested additives had limited impact on oxidative stress, although GPx and CAT activities were reduced in fish fed NUC and PHY, respectively. The experimental diets did not affect the immunological status of the fish, but further studies under an environment challenge are suggested to better evaluate their full potential.

Conflict of interest

The authors declare no conflict of interest.

Authors contributions

L.M.P.V., R.P., H.A., M.P. and K.V.: Conceptualization; Methodology; S.B., C.R., R.P., B.O., F.T. and M.M.: Investigation and Formal analysis; I.G. and L.B.: Formal analysis; L.M.P.V.: Funding acquisition, Visualization; All authors: Writing - Review & Editing.

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Table 1 - Formulation and proximate composition of the experimental diets.

	CTRL	*GRA*	*GRAP*	*PHY*	*AOS*	*NUC*
Ingredients (%)						
Fishmeal 70 [1]	4.1	4.1	4.1	4.1	4.1	4.1
Fishmeal 60 [2]	16.5	13.4	13.4	13.4	13.4	13.4
Soy protein concentrate [3]	8.3	8.3	8.3	8.3	8.3	8.3
Pea protein concentrate [4]	1.9	1.9	1.9	1.9	1.9	1.9
Wheat gluten [5]	12.1	12.1	12.1	12.1	12.1	12.1
Corn gluten [6]	6.6	6.6	6.6	6.6	6.6	6.6
Soybean meal [7]	11.1	11.1	11.1	11.1	11.1	11.1
Rapeseed meal [8]	4.1	4.1	4.1	4.1	4.1	4.1
Wheat meal [9]	13.8	8.4	8.4	8.4	5.9	8.3
Sardine oil [10]	12.0	12.2	12.2	12.2	12.2	12.2
Vit & Min Premix [11]	0.8	0.8	0.8	0.8	0.8	0.8
Binder [12]	0.2	0.2	0.2	0.2	0.2	0.2
L-Tryptophan [13]	0.1	0.1	0.1	0.1	0.1	0.1
DL-Methionine [14]	0.4	0.4	0.4	0.4	0.4	0.4
Hemoglobin powder [15]	5.0	5.0	5.0	5.0	5.0	5.0
Porcine gelatin [16]	3.0	3.0	3.0	3.0	3.0	3.0
Gracilaria gracilis [17]		8.3		8.3	8.3	8.3
Processed *G. gracilis* [18]			8.3			
Phytogenic compounds [19]				0.02		
Alginate oligosaccharide [20]					2.5	
Nucleotides [21]						0.08
Chemical composition						
Dry matter (DM, %)	89.5	92.2	91.0	91.4	91.4	93.0
Crude protein (% DM)	53.7	53.3	53.7	53.3	53.0	53.4
Crude fat (% DM)	16.9	17.4	17.5	17.6	17.8	17.1
Carbohydrates (% DM) [22]	22.1	20.4	19.7	20.3	19.3	20.5
Neutral detergent fiber (% DM)	25.3	12.9	15.3	12.4	14.0	14.6
Crude fiber (% DM)	1.3	1.7	1.7	1.6	1.7	1.7
Gross Energy (kJ g^{-1} DM)	22.7	22.3	22.3	22.2	22.0	22.2
Phosphorus (% DM)	0.9	0.9	0.9	0.9	0.9	0.9
Ash (% DM)	7.3	8.9	9.1	8.8	9.9	9.0

[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] Sardine oil: Sopropêche, France;
[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, 20000 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] L-Tryptophan, Feed grade amino acids, SORGAL, SA. Ovar, Portugal;

[14] DL-Metionine: 99% Evonik, Degussa GmbH, Germany;

[15] Porcine hemoglobin powder 92% CP, SONAC, The Netherlands;

[16] Lapi, Italy;

[17] *Gracilaria* sp.: 30.0% CP, 0.9% CF, 29.7% Neutral detergent fiber; 5.2% crude fiber; Algaplus Lda, Portugal;

[18] Physically processed *Gracilaria* sp.: 31.3% CP, 0.9% CF, 12.4% Neutral detergent fiber; 4.8% crude fiber; Algaplus Lda, Portugal;

[19] Digestarom® P.E.P. MGE150 – BIOMIN, Austria;

[20] >55% sodium oligoalginate, 9% moisture (< 30% sodium sulfate); oligo-alginate may not be retrieved by standard fiber methods, even if they should be considered as soluble dietary fibers; CEVA, France;

[21] NUCLEOFORCE FISH™, BIOIBERICA, Spain.

[22] Calculated by estimation, 100 - (ash + crude protein + crude fat)

Table 2 - Growth performance, whole body and muscle composition of European seabass fed the experimental diets for 106 days.

	CTRL[1]	*GRA*[1]	*GRAP*	*PHY*	*AOS*	*NUC*	*P value*
Growth and nutrient utilization							
Final body weight (g)	80.9 ± 2.0	83.1 ± 3.8	86.5 ± 3.8	85.5 ± 2.6	87.7 ± 3.9	81.1 ± 0.2	0.123
Weight gain (g)	51.3 ± 2.0	53.4 ± 3.7	56.8 ± 3.8	55.9 ± 2.6	58.0 ± 3.9	51.4 ± 0.2	0.122
Specific Growth Rate (SGR)	1.0 ± 0.02	1.0 ± 0.04	1.0 ± 0.04	1.0 ± 0.03	1.0 ± 0.04	1.0 ± 0.002	0.114
Voluntary feed intake (% ABW d^{-1})	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.03	1.4 ± 0.04	1.5 ± 0.04	1.5 ± 0.1	0.467
Feed conversion ratio	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	0.225
Somatic indexes							
Hepatosomatic index	2.1 ± 0.3^{a}	1.7 ± 0.3^{b}	1.9 ± 0.2^{ab}	1.7 ± 0.3^{b}	1.7 ± 0.4^{b}	1.8 ± 0.3^{b}	<0.001
Viscerosomatic index	5.0 ± 1.1	4.8 ± 0.8	4.9 ± 0.9	4.7 ± 0.9	4.7 ± 1.5	4.5 ± 0.6	0.679
Final whole body composition (% WW)[2]							
Dry matter	31.7 ± 0.7	32.0 ± 0.8	32.0 ± 0.6	30.9 ± 1.0	31.2 ± 0.6	30.8 ± 1.3	0.348
Protein	17.3 ± 0.3	17.7 ± 0.2	17.8 ± 0.3	17.8 ± 0.1	17.6 ± 0.3	17.5 ± 0.004	0.099
Fat	10.5 ± 1.0	11.0 ± 1.3	10.4 ± 1.2	8.7 ± 1.7	9.8 ± 1.0	9.4 ± 0.8	0.343
Energy (kJ g^{-1})	7.8 ± 0.3	7.9 ± 0.4	8.0 ± 0.2	7.5 ± 0.4	7.6 ± 0.2	7.5 ± 0.4	0.321
Ash	4.1 ± 0.4	3.9 ± 0.4	4.1 ± 0.2	4.1 ± 0.4	4.1 ± 0.1	4.2 ± 0.4	0.960
Fillet muscle composition (% WW)							
Moisture	74.6 ± 1.2^{b}	75.7 ± 0.7^{ab}	75.7 ± 0.9^{ab}	76.2 ± 0.5^{a}	76.2 ± 1.1^{a}	75.5 ± 1.1^{ab}	0.001
Crude protein	21.5 ± 0.3	20.9 ± 1.7	21.2 ± 0.3	21.3 ± 0.1	21.3 ± 0.4	21.3 ± 0.7	0.948
Crude fat	3.5 ± 0.1	2.9 ± 0.5	3.1 ± 0.6	2.3 ± 0.2	2.7 ± 0.3	2.8 ± 0.7	0.103

Values are means ± standard deviation.
Means in a row that do not share a common superscript letter indicate significant differences ($p<0.05$).
[1] Values adapted from Batista et al. (2020b).
[2] Initial body composition: dry matter, 35.5%; protein, 18.8%; lipids, 12.0%; energy, 8.2 kJ g^{-1}; ash, 4.5%.

Table 3 – Apparent digestibility coefficient (ADC) and nutrient balance of the experimental diets fed to European seabass for 106 days.

	CTRL[1]	*GRA*[1]	*GRAP*	*PHY*	*AOS*	*NUC*	*P value*
Diets ADCs (%)							
Dry matter	50.1 ± 5.6^{a}	16.0 ± 2.1^{cd}	43.6 ± 4.3^{ab}	13.4 ± 5.3^{d}	24.8 ± 8.7^{cd}	31.1 ± 2.9^{bc}	<0.001
Protein	85.4 ± 0.4^{a}	67.7 ± 8.1^{c}	83.9 ± 1.9^{ab}	73.3 ± 1.3^{bc}	77.2 ± 3.3^{abc}	78.3 ± 4.2^{abc}	0.002
Lipids	89.4 ± 1.0^{ab}	86.3 ± 6.7^{b}	94.6 ± 0.6^{a}	92.5 ± 0.7^{ab}	93.2 ± 0.6^{ab}	91.4 ± 2.1^{ab}	0.025
Energy	63.8 ± 2.0^{a}	37.6 ± 5.2^{b}	63.5 ± 3.0^{a}	43.2 ± 6.6^{b}	47.4 ± 8.1^{b}	53.2 ± 6.1^{ab}	<0.001
Phosphorus	58.5 ± 7.2	51.2 ± 2.9	59.3 ± 3.9	56.9 ± 4.4	58.1 ± 4.6	51.9 ± 0.5	0.240
Nitrogen (N) balance *(mg 100 g ABW^{-1} d^{-1})*							
Digestible N intake	102.8 ± 2.1^{a}	85.3 ± 12.7^{b}	105.5 ± 4.0^{a}	89.6 ± 3.2^{a}	97.0 ± 6.8^{a}	99.9 ± 8.6^{a}	0.034
N gain	22.9 ± 0.6^{b}	24.4 ± 0.7^{ab}	25.4 ± 1.5^{a}	25.3 ± 0.4^{a}	25.2 ± 0.4^{a}	23.4 ± 0.1^{ab}	0.009
NRE (% Digestible N)	22.3 ± 0.7^{b}	29.0 ± 4.1^{a}	24.1 ± 1.3^{ab}	28.3 ± 1.3^{ab}	26.1 ± 2.0^{ab}	23.5 ± 2.0^{ab}	0.021
Fecal N losses	17.6 ± 0.8^{c}	40.5 ± 9.0^{a}	20.2 ± 2.1^{bc}	32.6 ± 1.6^{ab}	28.6 ± 3.4^{abc}	27.5 ± 4.4^{abc}	0.001
Non-fecal N losses	79.9 ± 2.1^{a}	61.0 ± 12.5^{b}	80.1 ± 3.6^{a}	64.3 ± 3.4^{a}	71.8 ± 6.8^{a}	76.5 ± 8.5^{a}	0.026
Phosphorus (P) balance *(mg 100 g ABW^{-1} d^{-1})*							
Digestible P intake	7.2 ± 0.8	6.7 ± 0.4	7.6 ± 0.4	7.2 ± 0.7	7.6 ± 0.7	6.8 ± 0.3	0.346
P gain	5.9 ± 0.8	5.6 ± 0.6	6.0 ± 0.3	6.5 ± 1.5	6.3 ± 0.4	6.3 ± 1.5	0.650
PRE (% Digestible P)	82.6 ± 12.2	83.8 ± 12.0	79.0 ± 0.8	91.0 ± 25.3	82.6 ± 10.9	92.2 ± 26.0	0.962
Fecal P losses	5.1 ± 0.9	6.4 ± 0.5	5.2 ± 0.6	5.5 ± 0.5	5.5 ± 0.6	6.3 ± 0.1	0.193
Non-fecal P losses	1.3 ± 0.9	1.1 ± 0.9	1.6 ± 0.1	1.0 ± 1.7	1.4 ± 1.0	0.9 ± 1.3	0.970
Energy (E) balance *(kJ kg ABW^{-1} d^{-1})*							
Digestible E intake	202.9 ± 7.3^{a}	123.9 ± 20.5^{b}	207.2 ± 11.5^{a}	137.9 ± 20.8^{b}	154.9 ± 29.3^{ab}	176.3 ± 25.8^{ab}	0.002
Metabolizable E	183.0 ± 7.1^{a}	108.7 ± 17.4^{b}	187.3 ± 11.0^{a}	121.9 ± 20.3^{b}	137.0 ± 27.8^{ab}	157.2 ± 23.7^{ab}	0.001
E gain	66.1 ± 4.8	68.4 ± 7.6	72.2 ± 4.5	64.7 ± 4.4	67.8 ± 4.7	61.7 ± 5.0	0.384
ERE (% Digestible E)	32.5 ± 1.2^{c}	56.4 ± 11.4^{a}	34.8 ± 0.5^{bc}	47.4 ± 5.2^{ab}	44.5 ± 5.4^{ab}	35.6 ± 8.1^{bc}	0.028
Fecal E losses	114.9 ± 7.1^{c}	205.1 ± 11.4^{a}	118.8 ± 9.2^{c}	181.1 ± 21.4^{ab}	171.0 ± 22.9^{ab}	154.8 ± 15.0^{bc}	<0.001
Non-fecal E losses	19.9 ± 0.5^{a}	15.2 ± 3.1^{b}	19.9 ± 0.9^{a}	16.1 ± 0.9^{ab}	17.9 ± 1.7^{ab}	19.0 ± 2.1^{ab}	0.026
Total heat production	117.0 ± 2.7^{a}	40.3 ± 20.3^{c}	115.1± 6.5^{a}	57.3 ± 17.5^{bc}	69.2 ± 23.3^{abc}	95.6 ± 28.7^{ab}	0.001

Values are means ± standard deviation; n=3; Means in a row that do not share a common superscript letter indicate significant differences ($p<0.05$).
RE, retention efficiency. [1] Values adapted from Batista et al. (2020b).

Table 4 - Intestinal morphology of European sea bass.

	***CTRL*[1]**	***GRA*[1]**	***GRAP***	***PHY***	***AOS***	***NUC***	***P value***
Anterior intestine							
Cross sectional area (mm²)	5.7 ± 1.0	5.6 ± 1.4	7.1 ± 1.3	6.4 ± 1.4	4.6 ± 1.1	5.9 ± 1.8	0.081
Villus length (mm)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.2	0.913
Villus width (μm)	131.6 ± 16.5^{ab}	113.2 ± 9.4^{b}	150.6 ± 6.2^{a}	110.6 ± 12.2^{b}	131.9 ± 18.5^{ab}	127.8 ± 20.2^{ab}	0.002
Muscularis externa (μm)	98.7 ± 18.5	107.8 ± 26.6	117.5 ± 18.7	114.3 ± 26.3	97.1 ± 10.1	87.2 ± 12.8	0.105
Outer longitudinal layer	32.8 ± 3.9	40.2 ± 11.0	42.9 ± 6.8	40.4 ± 11.4	35.9 ± 4.9	31.8 ± 5.2	0.126
Inner circular layer	62.5 ± 11.2	67.6 ± 18.0	74.6 ± 12.3	67.9 ± 7.5	61.1 ± 5.8	55.4 ± 8.0	0.101
Goblet cells (nº GC fold^{-1})	55.3 ± 15.8^{ab}	65.6 ± 14.1^{a}	73.2 ± 26.8^{a}	66.3 ± 17.5^{a}	38.9 ± 7.8^{b}	46.4 ± 7.9^{ab}	0.010
Neutral GC	8.9 ± 4.2	7.5 ± 3.5	11.6 ± 9.2	8.4 ± 4.5	10.4 ± 7.9	7.2 ± 3.1	0.996
Acid GC	46.4 ± 13.7^{ab}	58.1 ± 16.1^{a}	61.6 ± 20.5^{a}	57.9 ± 15.0^{a}	30.7 ± 6.4^{b}	44.2 ± 13.8^{ab}	0.018
Posterior intestine							
Cross sectional area (mm²)	3.1 ± 0.7	4.2 ± 0.6	4.3 ± 0.3	4.2 ± 1.1	4.9 ± 2.2	4.8 ± 1.4	0.294
Villus length (mm)	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.265
Villus width (μm)	98.8 ± 9.5	97.2 ± 9.4	107.9 ± 10.7	109.8 ± 3.0	104.8 ± 15.0	112.6 ± 18.3	0.243
Muscularis externa (μm)	70.3 ± 15.4	75.3 ± 27.5	94.2 ± 16.6	102.4 ± 31.4	90.4 ± 22.3	99.5 ± 3.9	0.126
Outer longitudinal layer	33.5 ± 12.3	29.0 ± 10.5	39.2 ± 6.6	38.9 ± 14.7	36.8 ± 11.2	40.4 ± 8.9	0.542
Inner circular layer	41.1 ± 8.5	46.4 ± 17.1	55.0 ± 11.2	63.6 ± 17.2	53.7 ± 11.2	59.0 ± 2.7	0.088
Goblet cells (nº GC fold^{-1})	81.0 ± 24.6	100.7 ± 15.0	73.7 ± 15.2	104.4 ± 40.5	77.4 ± 29.4	100.2 ± 41.8	0.306
Neutral GC	5.6 ± 5.5	0.9 ± 0.7	0.5 ± 0.3	1.1 ± 0.8	1.0 ± 0.6	5.4 ± 5.4	0.019
Acid GC	75.5 ± 22.4	99.8 ± 15.1	72.9 ± 14.8	103.3 ± 40.0	76.4 ± 29.2	107.9 ± 31.1	0.105

Values are means ± standard deviation; n = 6.

Means in a row that do not share a common superscript letter indicate significant differences ($p<0.05$).

[1] Values adapted from Batista et al. (2020b).

Table 5 - Specific activity (μm min^{-1} g^{-1} tissue) of maltase, sucrose-isomaltase (SI), intestinal alkaline phosphatase (IAP) and ɤ-glutamyltransferase (ɤGT) in European sea bass intestinal trait after five hours fasting.

	CTRL[1]	*GRA*[1]	*GRAP*	*PHY*	*AOS*	*NUC*	*P value*
Pyloric caeca							
Maltase	2770.2 ± 251.9^{ab}	2441.4 ± 364.5^{abc}	2511.5 ± 465.7^{abc}	2225.6 ± 416.7^{bc}	2138.5 ± 618.6^{c}	2936.5 ± 776.8^{a}	0.003
SI	1023.3 ± 159.3^{ab}	822.3 ± 226.2^{b}	740.7 ± 188.1^{b}	762.7 ± 194.1^{b}	773.2 ± 194.1^{b}	1119.6 ± 377.8^{a}	0.001
IAP	68.1 ± 10.1^{ab}	82.3 ± 13.7^{ab}	62.9 ± 16.2^{b}	67.1 ± 22.3^{ab}	88.0 ± 25.8^{a}	78.3 ± 24.4^{ab}	0.020
ɣGT	1.0 ± 0.2	1.2 ± 0.2	1.2 ± 0.5	0.8 ± 0.3	1.0 ± 0.4	1.2 ± 0.4	0.080
Anterior intestine							
Maltase	5793.5 ± 1444.7^{ab}	4257.8 ± 1162.5^{bc}	4571.3 ± 571.5^{abc}	4211.8 ± 1077.1^{c}	6082.2 ± 1350.2^{a}	5621.3 ± 1660.0^{abc}	0.001
SI	2238.5 ± 547.5^{a}	1499.0 ± 362.2^{b}	1618.2 ± 336.9^{ab}	1598.6 ± 582.4^{ab}	2096.7 ± 739.7^{ab}	1981.0 ± 728.35^{ab}	0.010
IAP	123.6 ± 30.4^{ab}	115.3 ± 40.0^{b}	105.9 ± 23.5^{b}	104.7 ± 33.3^{b}	174.4 ± 69.4^{a}	120.6 ± 44.0^{b}	0.004
ɣGT	1.5 ± 0.8	1.3 ± 0.5	1.4 ± 0.4	1.5 ± 0.4	1.4 ± 0.4	1.4 ± 0.5	0.920
Posterior intestine							
Maltase	4633.2 ± 944.2^{ab}	4472.5 ± 958.0^{ab}	5695.3 ± 1897.8^{a}	3606.5 ± 1068.1^{b}	5509.3 ± 1404.3^{a}	3972.1 ± 941.4^{b}	<0.001
SI	1821.7 ± 560.7	1671.1 ± 462.9	2014.4 ± 1238.4	1638.3 ± 598.7	1976.8 ± 573.5	1420.7 ± 255.5	0.290
IAP	95.3 ± 21.1	91.1 ± 34.1	109.3 ± 25.5	91.1 ± 34.1	115.0 ± 42.7	84.0 ± 30.3	0.170
ɣGT	7.9 ± 3.7^{b}	8.0 ± 4.6^{b}	14.3 ± 8.0^{a}	7.2 ± 3.2^{b}	7.1 ± 1.9^{b}	3.5 ± 1.7^{b}	<0.001

Values are means ± standard deviation: n = 12.
Means in a row that do not share a common superscript letter indicate significant differences ($p<0.05$).
[1] Values adapted from Batista et al. (2020b).

Table 6 – Plasma metabolite levels and innate immune parameters in European sea bass after 24 h of fasting.

	***CTRL*[1]**	***GRA*[1]**	***GRAP***	***PHY***	***AOS***	***NUC***	*P value*
Metabolite levels							
Glucose (mg dL^{-1})	120.9 ± 21.5	149.6 ± 26.3	122.7 ± 28.6	138.9 ± 34.1	129.5 ± 23.1	142.4 ± 24.3	0.062
Total cholesterol (mg dL^{-1})	169.4 ± 31.2^{a}	187.3 ± 22.1^{a}	162.2 ± 32.7^{ab}	161.2 ± 13.6^{ab}	160.7 ± 34.9^{ab}	140.6 ± 20.1^{b}	0.001
Triglycerides (mg dL^{-1})	436.8 ± 146.6^{a}	316.2 ± 88.5^{bc}	298.5 ± 110.9^{bc}	273.7 ± 53.0^{bc}	237.7 ± 53.0^{c}	379.8 ± 138.6^{ab}	<0.001
Total protein (mg dL^{-1})	3.9 ± 0.8	4.2 ± 0.9	4.0 ± 0.7	4.1 ± 0.4	4.0 ± 0.6	4.1 ± 0.6	0.891
Albumin (mg dL^{-1})	1.0 ± 0.2^{ab}	1.1 ± 0.1^{a}	1.0 ± 0.2^{ab}	0.9 ± 0.1^{ab}	0.9 ± 0.2^{b}	0.8 ± 0.1^{b}	0.010
Innate immune parameters							
Lysozyme (EU mL^{-1})	834.7 ± 283.4	903.7 ± 362.3	956.1 ± 181.2	974.7 ± 225.1	836.6 ± 347.2	823.7 ± 388.7	0.762
Peroxidase (EU mL^{-1})	301.4 ± 106.8	279.9 ± 123.3	322.1 ± 131.7	324.4 ± 161.9	283.8 ± 139.5	182.3 ± 67.2	0.103
ACH50 (Units mL^{-1})	238.7 ± 101.4	153.9 ± 61.3	197.5 ± 51.5	188.9 ± 96.2	179.0 ± 75.2	219.8 ± 92.6	0.201

Values are means ± standard deviation; n = 12.
Means in a row that do not share a common superscript letter indicate significant differences ($p<0.05$).
[1] Values adapted from Batista et al. (2020b).

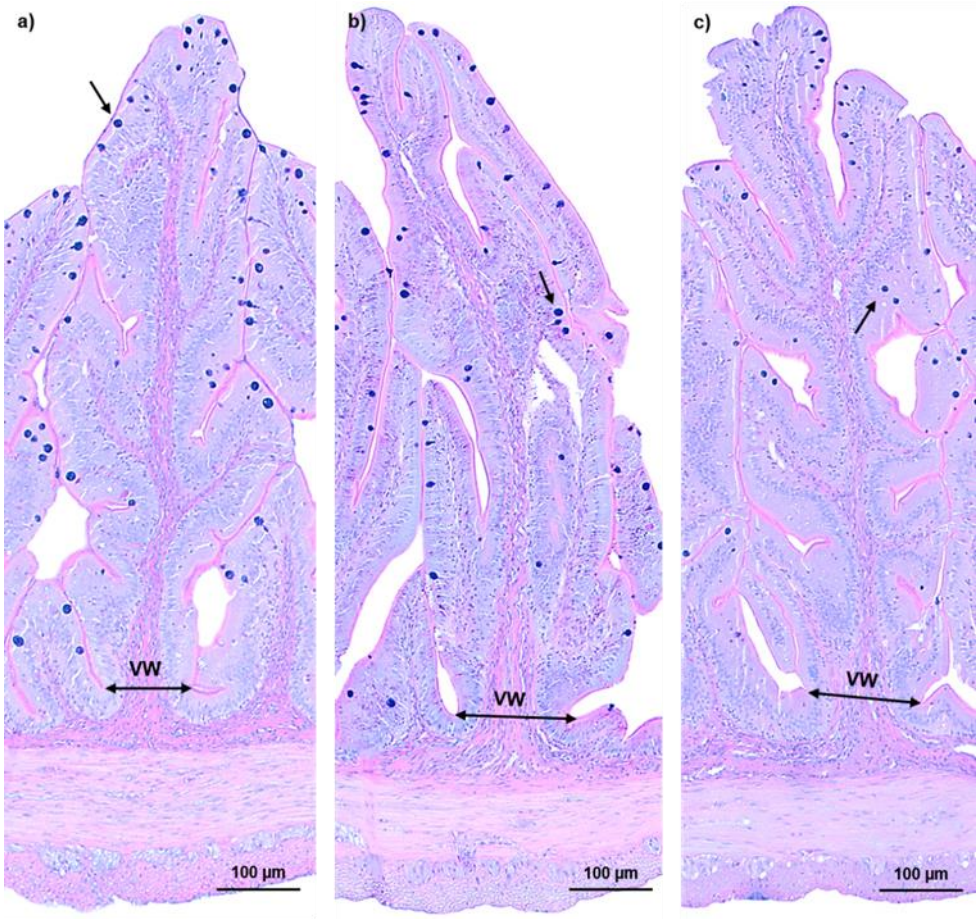

Figure 1– Histological sections (Alcian Blue/PAS staining) of the anterior intestine of European sea bass fed the following diets: a) GRA; b) GRAP and c) AOS. Note the reduced VW, v*illus* width, in fish fed GRA diet compared to fish fed GRAP and the higher number of acid goblet cells (in blue; arrows) in fish fed GRA and GRAP diets compared to fish fed AOS diet.

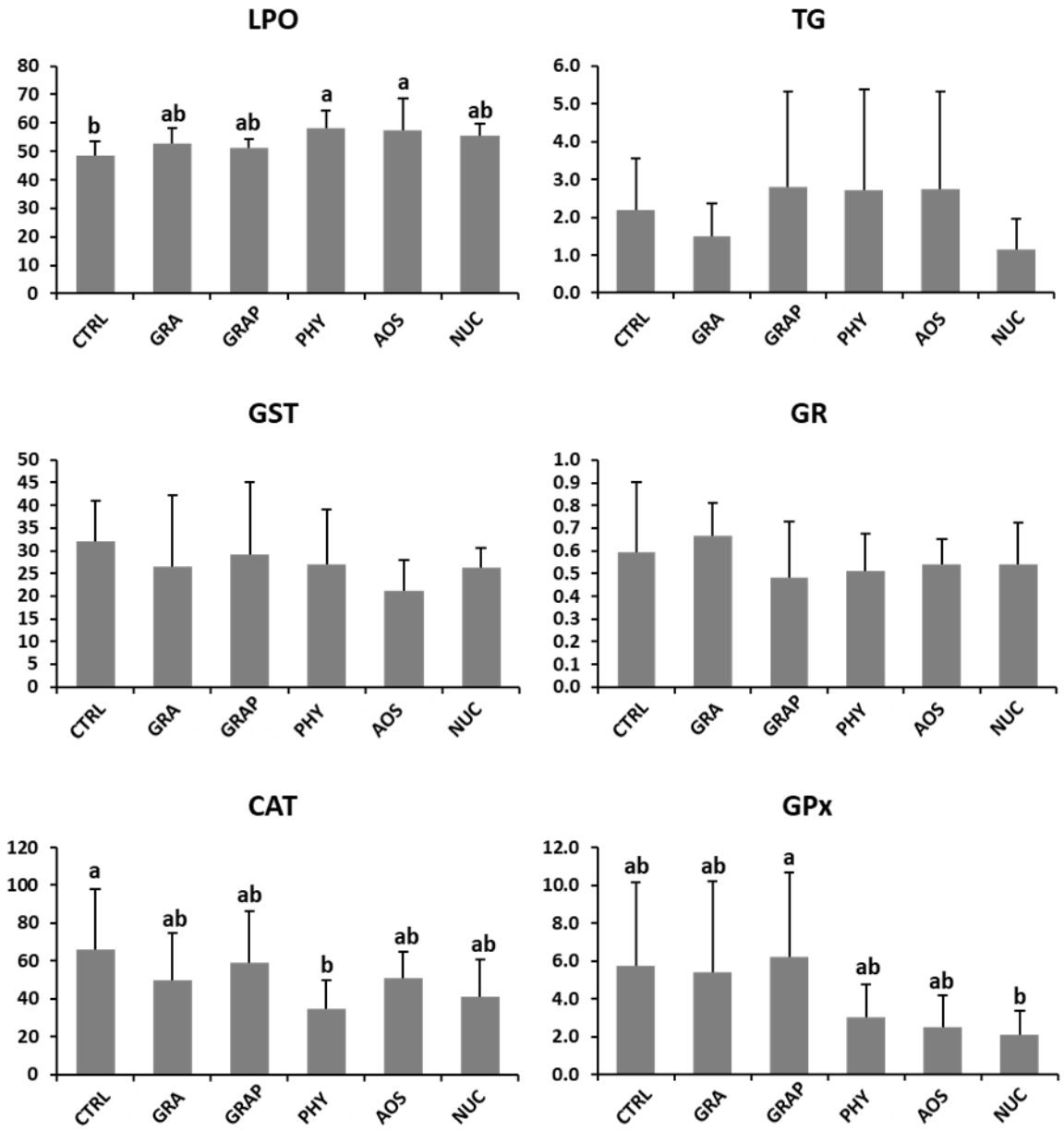

Figure 2 – Oxidative stress parameters (LPO expressed in nmol TBARS g tissue^{-1}; TG, GPx, GST and GR expressed in nmol min^{-1} mg protein^{-1}; and CAT expressed in µmol min^{-1} mg protein^{-1}) determined in the liver of European sea bass after 106 days of feeding. Values are means ± standard deviation: n = 12; For each parameter, bars without a common letter differ significantly ($p<0.05$). Values from CTRL and GRA were adapted from Batista et al. (2020b). TG, Total glutathione; GPx, Glutathione peroxidase; GST, Glutathione S-Transferase; GR, Glutathione reductase; CAT, Catalase; LPO, Lipid peroxidation.

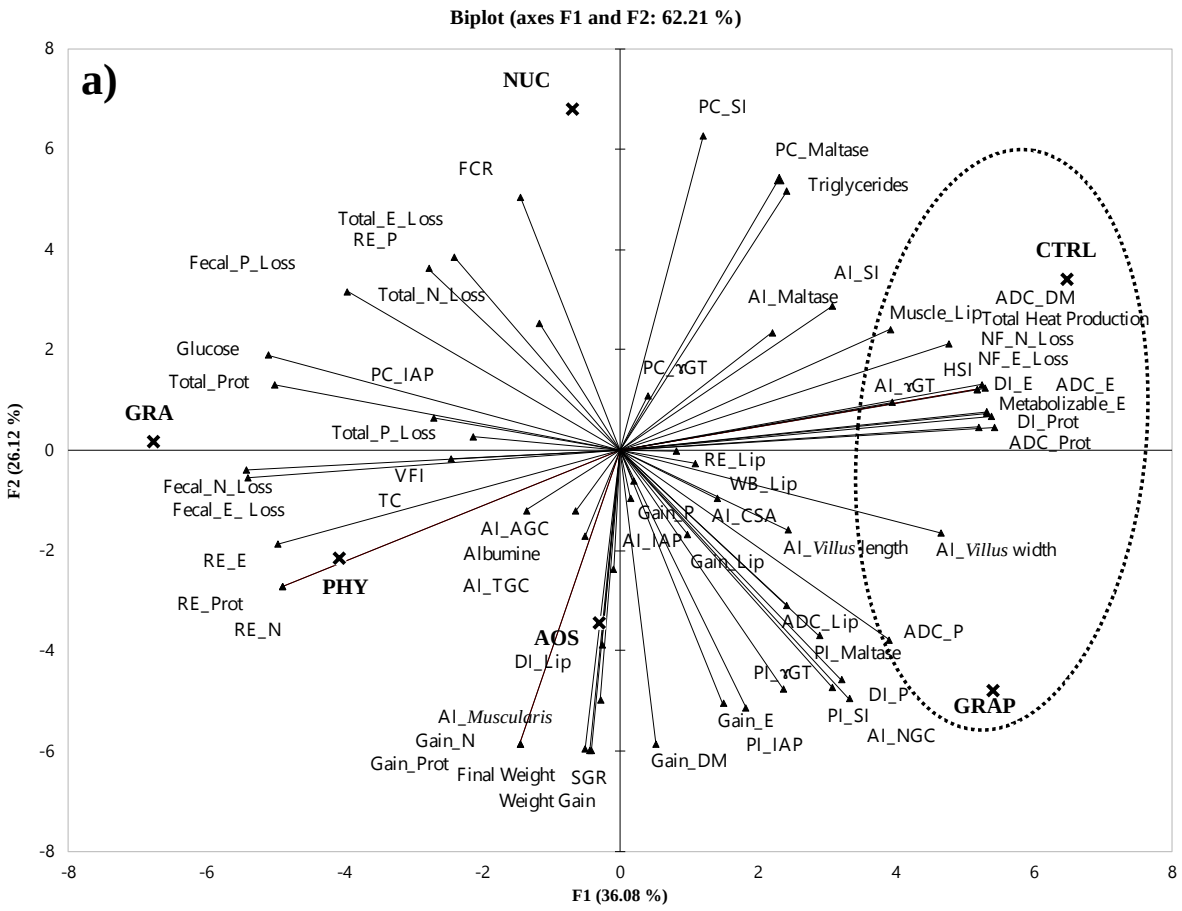
Biplot (axes F1 and F2: 62.21 %)
a)
NUC
PC_SI
PC_Maltase
Triglycerides
FCR
Total_E_Loss
RE_P
Fecal_P_Loss
Total_N_Loss
CTRL
AI_SI
AI_Maltase
ADC_DM
Muscle_Lip
Total Heat Production
NF_N_Loss
Glucose
PC_IAP
PC_γGT
HSI
NF_E_Loss
Total_Prot
AI_γGT
DI_E
ADC_E
GRA
Metabolizable_E
Total_P_Loss
DI_Prot
ADC_Prot
RE_Lip
VFI
WB_Lip
Fecal_N_Loss
TC
Gain_P
AI_CSA
Fecal_E_ Loss
AI_AGC
AI_IAP
AI_Villus length
AI_Villus width
Albumine
Gain_Lip
RE_E
PHY
AI_TGC
RE_Prot
ADC_Lip
ADC_P
RE_N
AOS
PI_Maltase
DI_Lip
PI_γGT
DI_P
GRAP
AI_Muscularis
Gain_E
PI_SI
Gain_N
PI_IAP
AI_NGC
Gain_Prot
Final Weight
SGR
Gain_DM
Weight Gain
F2 (26.12 %)
F1 (36.08 %)
Biplot (axes F1 and F2: 62.67 %)
b)
AI_Maltase
AI_SI
PC_SI
AI_IAP
PC_IAP
AOS
NUC
PC_Maltase
CTRL
DI_Prot
ADC_DM
ADC_Prot
AI_γGT
ADC_E
DI_E
PC_γGT
AI_Villus width
PI_Maltase
DI_P
PI_IAP
DI_Lip
ADC_P
ADC_Lip
PI_SI
AI_Villus length
AI_NGC
GRA
PHY
GRAP
PI_γGT
AI_CSA
AI_AGC
AI_TGC
AI_Muscularis
F2 (25.89 %)
F1 (36.78 %)

Figure 3 – A: Principal component analysis biplot based on the mean scores and loadings of all the variables measured in European sea bass fed the experimental diets (CTRL, GRAP, GRA, PHY, AOS, NUC); B: Principal component analysis with ADC, digestible intake, intestine morphology and the specific activity of intestinal enzymes as the variables. DM, dry matter; E, energy; Prot, Protein; Lip, Lipids; P, Phosphorus; N, nitrogen; NF, non-fecal; RE, retention efficiency; DI, digestible intake; ADC, apparent digestibility coefficient; FCR, feed conversion ratio; SGR-specific growth rate; VFI, voluntary feed intake; WB, whole-body; TC, total cholesterol; AI, anterior intestine; PI, posterior intestine; PC, pyloric caeca; HSI, hepatosomatic index; TGC, total goblet cells; NGC, neutral goblet cells; AGC, acid goblet cells; CSA, Cross sectional area; *Muscularis*, *Muscularis* externa; SI, sucrose-isomaltase; IAP, intestinal alkaline phosphatase; ɤGT, ɤ-glutamyltransferase.

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