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Antioxidant, metabolic and digestive biomarker responses of farmed *Sparus aurata* supplemented with *Laminaria digitata*

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ABSTRACT

The present study aimed to investigate the effectiveness of supplemented diets with the brown macroalga *Laminaria digitata* in improving the antioxidant, metabolic and digestive performance in gilthead seabream (*Sparus aurata)*, using a multi-biomarker approach. Three experimental diets supplemented with 1.5 %, 3 % and 6 % of dried powdered *L. digitata* were tested against a control diet. After 30 and 60 days, *S. aurata* juveniles were sampled and muscle, gut and liver tissues were collected. Results showed that fish fed with 1.5 % *L. digitata* exhibited higher specific growth rate (SGR) after 30 days. Additionally, at this supplementation level, there was a significant reduction in antioxidant enzyme activities (catalase, CAT; glutathione S-transferase, GST; superoxide dismutase, SOD) and in lipid peroxidation (LPO) in muscle and liver tissues. In contrast, gut antioxidant enzyme activity increased with higher macroalga concentrations (3 % and 6 %). Fish fed with 6 % *L. digitata* revealed a significant decrease in muscle aerobic potential (citrate synthase (CS) activity) but increased anaerobic capacity (lactate dehydrogenase (LDH) activity). Moreover, the 1.5 % *L. digitata* supplemented diets led to a significant increase in amylase activity after 30 days. After 60 days, fed with 6 % *L. digitata* showed lower hepatosomatic index (HSI) compared to animals fed on control diet. Additionally, trypsin activity was higher in fish fed the supplemented diets, especially at 3 % *L. digitata*. Pepsin activity was significantly supressed in fish fed diets with higher macroalga concentrations (3 % and 6 %). Overall, the current findings highlight the beneficial effects with lower doses of L. *digitata* on farmed marine fish performance and welfare. However, additional research is needed to establish the most cost-effective seaweed supplementation dose and validate this strategy at an industrial scale.

1. Introduction

Aquaculture is the sector with the fastest expansion rate within animal production, consistently increasing its share of the total seafood produced worldwide every year (FAO, 2018). It plays a crucial role in food security, being responsible for more than 50 % of global seafood production and thus contributing to end hunger and malnutrition of the world's growing population (FAO, 2022). Still, aquaculture's expansion is strongly dependent upon the availability and costs of aquafeeds and/or the raw materials that compose them. Although the use of meals and oils derived from wild fish species in aquafeeds has been consistently decreasing, these are still important feed components for many carnivorous fishes due to their high content of protein and lipids and its digestibility and palatability (Estruch et al., 2018; Turchini et al., 2019). Yet, marine fish meal and oils are considered environmentally unsustainable since they are finite ingredients and involve long transportation distances and habitat degradation (Malcorps et al., 2019). Since 2022, the sector was further defied by the scarcity and escalating prices of plant-based raw materials (such as cereals/vegetable meals and oils) that came along with the war in Ukraine. Hence, over the last decade,

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the aquafeed industry has evidenced an increasing demand for low-cost and more sustainable ingredients, prompting the exploration of new sources of nutrients and bioactive compounds (BAC) that can be potentially used in formulas that meet the nutritional requirements and enhance the performance of farmed fish species (FAO, 2018).

Seaweeds have been pointed out as a suitable ingredient containing relevant amounts of nutrients (e.g., minerals, vitamins, antioxidants, and polyunsaturated fatty acids) that are not only essential for aquafeed formulations but also in the human diet (Castanho et al., 2017; Pereira et al., 2019; Morais et al., 2020; Costa et al., 2021; Mota et al., 2023). They are also abundant in BACs that can be converted into a wide array of secondary metabolites with diverse biological activities (Gupta and Abu-Ghannam, 2011; Leandro et al., 2019). These compounds have been associated with a range of beneficial effects including enhanced growth performance, physiological activity, immune defence, improved antioxidant capacity and better stress response (Peixoto et al., 2016; Marques et al., 2019; Morais et al., 2020; Thépot et al., 2021; Thépot et al., 2022). Moreover, macroalgae are distinguished by their rapid growth rates and high biomass productivity, coupled with a low environmental impact. They do not compete with other crops for land, which makes them an environmentally sustainable alternative (Hull-Cantillo et al., 2022).

Several studies have investigated the dietary inclusion of different macroalgae in carnivorous fish diets and have shown that levels of inclusion up to 10 % resulted in beneficial effects or absence of adverse effects (; Norambuena et al., 2015; Peixoto et al., 2019; Passos et al., 2021). However, at higher inclusion levels, growth performance, feed efficiency and digestibility of diets have been negatively affected (Norambuena et al., 2015; Shpigel et al., 2017; Sotoudeh and Jafari, 2017; Batista et al., 2020). The literature supports that algae supplementation's effects on aquafeeds are species-specific and dose-dependent (Norambuena et al., 2015; Guerreiro et al., 2019). Moreover, the origin, form and potential interactions with other dietary components may also influence supplementation efficiency (Ribeiro et al., 2017; Saffari et al., 2017). In this context, determining the effective doses necessary to improve the welfare of farmed animals, often compromised by captivity, high rearing densities and disease outbreaks, remains the principal focus of most studies (Kamunde et al., 2019). Acquiring such knowledge is, thus, utmost important for the expansion of the aquaculture sector, as it can open the door for new raw materials with added value to be used within the aquafeeds industry, therefore, contributing to diminish the reliance upon unsustainable and/or costly feed ingredients (Kamunde et al., 2019).

Gilthead seabream (*Sparus aurata*) is one of the most important commercial-farmed species in Europe, being consumed mainly in the Mediterranean area and representing 10 % of the whole European aquaculture production (FAO, 2018; European Commission, 2019). As a carnivorous species, gilthead seabream typically requires a significant portion of fish meal and oil in aquafeeds (Estruch et al., 2018). Therefore, validating the use of macroalgae as a nutritious and functional ingredient of aquafeeds specifically designed for this farmed fish species would, most certainly, constitute a promising step towards upgrading sustainable aquaculture practices. The welfare assessment of fish is considered overly complex but is becoming increasingly important when evaluating new feed concepts (Carbonara et al., 2021). The current knowledge concerning the incorporation of brown seaweeds, in particular *Laminaria digitata*, into aquafeeds is very limited, and their potential nutraceutical and functional impacts on fish welfare, metabolism, and digestive responses have been little explored.

Hence, the present study aimed to investigate the effectiveness of aquafeeds supplemented with *Laminaria digitata,* a brown macroalga, at three different inclusion levels (1.5 %, 3 % and 6 %) in improving the antioxidant (catalase activity, glutathione S-transferase activity, superoxide dismutase activity and lipid peroxidation), metabolic (citrate synthase activity and lactate dehydrogenase activity) and digestive performance (amylase activity, pepsin activity, trypsin activity and lipase activity) of juvenile *Sparus aurata*, following a multi-biomarker approach that combines responses at the individual and biochemical levels.

2. Materials and methods

2.1. Experimental diets

Brown macroalgae *Laminaria digitata* were collected from Parc Naturel Marin d'Irose (West coast of Brittany, France). After the harvesting, macroalgae biomass was selected, milled and dried with bench-top fluid-bed technology (TG200, Retsch, Germany) by Algaia's factory (Lannilis, France). Four experimental functional diets (2.0-mm pellets) formulated to be isoenergetic (21.0 MJ/kg), isoproteic (46.1 % crude protein) and isolipidic (16.2 % crude fat), were manufactured and supplied by SPAROS Lda. (Olhão, Portugal). The control diet (CTR) used in this study did not include any macroalgae supplementation and was formulated according to the nutritional requirements of juvenile *Sparus aurata* (Table 1). The remaining experimental diets were supplemented with dried powdered L. *digitata,* at three distinct inclusion levels: 1.5 % (LD-1.5 %), 3 % (LD-3 %) and 6 % (LD-6 %), in detriment of wheat meal. Detailed feed composition is shown in Table 1.

The proximate chemical composition of the experimental diets (Table 1) was determined according to the methods described by the Association of Official Analytical Chemists (AOAC (Association of Official Analytical Chemists) International, 2005). Dry matter was measured by drying the samples at 105 °C for 24 h, while ash content was determined by combustion at 550 °C for 12 h. Crude protein was analysed using a flash combustion technique, followed by a gas chromatographic separation and thermal conductivity detection (nitrogen analyser FP428, LECO, USA), with a nitrogen conversion factor of 6.25. Lipid content was determined using the Soxhlet extration method.

2.2. Feeding trial and sampling

Gilthead seabream (*S. aurata*) specimens ($n = 108$) were reared until juvenile stage (total weight: 8.05 ± 0.6 g; total length: 8.2 ± 0.1 cm) in the Aquaculture Research Station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) using routine hatchery conditions. Juvenile fish were then transported to LABVIVOS (IPMA, Algés, Portugal) facilities, where they were maintained for 15 days in a quarantine system before entering the experimental setup. During quarantine, fish were hand-fed 2 % of their average body weight (BW) with CTR feed and no mortality was observed.

After acclimation, *S. aurata* specimens (total weight: 10.12 ± 0.8 g) were equally and randomly distributed in 12 rectangular-shaped glass tanks (total volume of 50 L each) within experimental recirculation aquaculture systems (RAS). To maintain seawater quality, each RAS was equipped with a protein skimmer (V^{2}Skim Pro Protein Skimmer 450, TMC Iberia, Portugal), a physical filter composed of a filter bag (400 μm; TMC Iberia, Portugal), filter sponge and glass wool, a biological filter (V^{2}Bio 1000 Fluidised Sand Bed Filter and Bio Balls 1.5″ Aquarium Pond Filter), a UV sterilizer (VE^{2}cton 200, TMC Iberia, Portugal), an automatic seawater refrigeration system (Foshan WEINUO Refrigeration Equipment Co., Ltd., China) and submerged digital thermostats (300 W, V2Therm Digital Heaters, TMC Iberia, Portugal) to control temperature, and submerged air stones in each tank to adjust dissolved oxygen. After 7 days of acclimation to the experimental setup, the feeding trial was initiated, during which four dietary treatments were carried out, each of them in triplicate (i.e., 3 tanks/treatment = 12 tanks total, $n = 9$ fish per replicate tank, 27 per treatment in total): i) control treatment (CTR), i.e. animals fed with CTR diet (without macroalgae supplementation); ii) LD-1.5 %, i.e. animals fed with aquafeed supplemented with 1.5 % of L. *digitata*; iii) LD-3 %, i.e. animals fed with aquafeed supplemented with 3 % of *L. digitata*; and iv) LD-6 %, i.e. animals fed with aquafeed supplemented with 6 % of *L. digitata*. Fish density was kept below 5 kg body

Table 1
Diet formulation and proximate composition of CTR and LD-enriched feeds (% dry matter). Abbreviations: CTR – control feed; LD-1.5 % – 1.5 % inclusion of *L. digitata*; LD-3 % – 3 % inclusion of *L. digitata*; LD-6 % – 6 % inclusion of *L. digitata*.

	Diets			
Ingredients (%)	**CTR**	**LD-1.5 %**	**LD-3 %**	**LD-6 %**
Fishmeal Super Prime[a]	22.0	22.0	22.0	22.0
Fish protein concentrate[b]	2.0	2.0	2.0	2.0
Laminaria digitata		1.5	3.0	6.0
Poultry meal[c]	5.0	5.0	5.0	5.0
Soy protein concentrate[d]	6.0	6.0	6.0	6.0
Pea protein concentrate[e]	2.0	2.0	2.0	2.0
Wheat gluten[f]	6.2	6.2	6.2	6.2
Corn gluten meal[g]	11.0	11.0	11.0	11.0
Soybean meal 44[h]	12.0	12.0	12.0	12.0
Sunflower meal[i]	4.0	4.0	4.0	4.0
Wheat meal[j]	9.2	7.7	6.2	3.2
Faba beans (low tannins)[k]	6.0	6.0	6.0	6.0
Vitamin and mineral premix[l]	1.0	1.0	1.0	1.0
Choline chloride 50 %[m]	0.2	0.2	0.2	0.2
Monoammonium phosphate[n]	1.0	1.0	1.0	1.0
Fish oil[o]	5.0	5.0	5.0	5.0
Soybean oil[p]	7.4	7.4	7.4	7.4
Proximate composition (%)				
Dry matter, DM (%)	94.30	94.10	93.90	94.00
Crude protein	46.23	46.14	46.06	45.88
Crude fat	16.11	16.15	16.19	16.28
Fiber	2.48	2.56	2.64	2.79
Starch	11.50	10.55	9.60	7.71
Ash	7.55	7.24	6.93	6.32
Gross energy (MJ/kg feed)	21.06	21.01	20.96	20.86

Minerals (g or mg/kg 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 middling's.

[a] Diamante, Pesquera Diamante, Peru (crude protein, CP: 66.3 % dry matter, DM; crude fat, CF: 11.5 % DM).

[b] CPSP90, Sopropêche, France (CP: 82.6 % DM; CF: 9.6 % DM).

[c] SAVINOR UTS, Portugal (CP: 62.4 % DM; CF: 12.5 % DM).

[d] Soycomil P, ADM, The Netherlands (CP: 62.2 % DM; CF: 0.7 % DM).

[e] Lysamine GPS, Roquette, France (CP: 78.1 % DM; CF: 8.3 % DM).

[f] VITAL, Roquette, France (CP: 80.4 % DM; CF: 5.8 % DM).

[g] COPAM, Portugal (CP: 61.2 % DM; CF: 5.2 % DM).

[h] Solvent extracted, Ribeiro & Sousa Lda, Portugal (CP: 43.8 % DM; CF: 3.5 % DM).

[i] Dehulled solvent extracted, AGP Slovakia, s.r.o., Slovakia (CP: 42.9 % DM; CF: 3.8 % DM).

[j] Molisur, Spain (CP: 11.7 % DM; CF: 1.6 % DM).

[k] Ribeiro & Sousa, Portugal (CP: 24.5 % DM; CF: 1.7 % DM).

[l] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha-tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotidin 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.

[m] ORFA, The Netherlands (CP: 24.5 % DM; CF: 1.7 % DM).

[n] Windmill AQUAPHOS, ALIPHOS, The Netherlands.

[o] Sopropêche, France (CF: 98.1 % DM).

[p] JC Coimbra, Portugal (CF: 98.6 % DM).

weight L^{-1} in each tank to avoid physiological stress related to high animal density. During the feeding trials, fish were hand-fed twice a day with a feed amount equivalent to 2 % of fish average BW (adjusted throughout the trial, according to fish weight gain) over a 30 and 60-day period with the respective feeds (CTR, LD-1.5 %, LD-3 % or LD-6 %) and kept under optimal growing conditions (dissolved oxygen = 7.2 ± 0.2 mg L^{-1}; temperature = 20.0 ± 0.5 °C; pH = 8.0 ± 0.1; salinity = 35 ± 0.5 ‰; photoperiod = 12 h light/12 h dark). Salinity, pH and dissolved oxygen were daily monitored using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany) and temperature using a portable precision thermometer (TFX 430, Ebro Electronic, Germany) and all parameters were adjusted whenever needed. Total ammonia, nitrites, and nitrates levels were checked daily through colorimetric tests (Tropic Marin, USA) and were maintained within optimal levels (except for nitrates which were kept <50 mg L^{-1}). Fish faeces were daily removed along with 25 % of seawater renewal in each tank. No mortality was observed in any of the treatments during the 60 days of feeding trial.

On days 30 (T30) and 60 (T60) of the feeding trials, 12 fish were randomly collected from each treatment (4 fish collected from each of the 3 replicate tanks that composed one treatment) and were euthanized by immersion for 10 min in tricaine methanesulfonate solution at 2000 mg L^{-1} (MS-222, Acros Organics, Belgium) buffered with sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, USA). Fish were fasted 24 h before every sampling event, to avoid sample contamination with faeces and indigested feed content within the digestive tract. All fish were measured (total length, TL) and weighed (W) and muscle, gut and liver tissues were collected (approximately 100 mg of tissue). Liver weight was registered for subsequent hepatosomatic index determination (HSI). Given the need to perform tissue homogenizations in different buffer solutions (according to the type of biomarkers), the tissues from 6 fish of every treatment were saved for antioxidant enzymes activity determinations, whereas the remaining 6 fish were used in the assessment of metabolic (muscle) and digestive (gut) enzymes activities. Samples were frozen and kept at −80 °C until performing biochemical biomarkers analyses.

2.3. *Growth performance calculations*

Fish condition was determined based on biometric parameters such as weight (W) and total length (TL) by the calculation of the Fulton's condition index, K (Ricker, 1975) as follows:

$$K = 100 \times \frac{W\ (g)}{TL^3\ (cm)}$$

Liver condition was calculated using the hepatosomatic index (HSI) with the following equation:

$$HSI\ (\%) = \frac{Liver\ weight\ (g)}{W\ (g)} \times 100$$

Specific growth rate (SGR) was determined to describe the growth rate of fish, based on the natural logarithm of body weight, using the final body weight (FBW) and the initial mean body weight (IBW) and the number of days of trial:

$$SGR\ \left(\%day^{-1}\right) = \frac{[\ln(FBW) - \ln(IBW)]}{Days} \times 100$$

Feed conversion ratio (FCR) was measured based on the quantity of feed fed and the fish weight gain during a certain period, with lower FCR values indicating higher efficiency:

$$FCR = \frac{Dry\ feed\ supplied}{Fish\ wet\ weight\ gained}$$

2.4. *Antioxidant enzyme activities and lipid peroxidation*

Samples were homogenized in ice-cold conditions in phosphate buffered saline (PBS, 0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 KH_2PO_4, pH = 7.40 ± 0.02; reagents from Sigma-Aldrich, Germany) using an Ultra-Turrax device (T10 basic, Ika, Germany). Crude homogenates were centrifuged in 1.5 mL microtubes at 10,000 × g for 10 min (4 °C) and afterwards, the supernatant was transferred to new microtubes and frozen (−80 °C) until further analysis. Enzyme activities were determined through well-established protocols previously optimized for fish tissues (Maulvault et al., 2017). All assays were adapted to 96-well microplates and were carried out using a microplate reader (Thermo Scientific Multiskan GO, USA). Each sample was analysed in triplicate.

The activity of catalase (CAT; EC 1.11.1.6) was determined based on the procedure described in Maulvault et al. (2017). Formaldehyde standards, with concentrations from 5 to 75 μM (Sigma Aldrich, Germany) were used to obtain a calibration curve. Absorbance was read at 540 nm and results were presented as nmol min^{-1} mg^{-1} protein.

Glutathione S-transferase (GST; EC 2.5.1.18) activity assay was carried out according to the procedure described in Maulvault et al. (2017), using 100 mM of 1-Chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich, Germany) as substrate. Absorbance was read at 340 nm every min for 6 min. The absorbance increase is directly proportional to GST activity, which was determined using the CDNB molar extinction coefficient of 5.3 mM^{-1} cm^{-1} and expressed as nmol min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined using nitroblue tetrazolium (NBT, Sigma Aldrich, Germany) and xanthine oxidase (XOD, Sigma Aldrich, Germany), according to the method described in Maulvault et al. (2017). Sample absorbances were read every min for 5 min at 560 nm and results were expressed as the percentage of enzyme inhibition.

Lipid peroxidation (LPO) assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978), following the method described in Madeira et al. (2016a, 2016b). A ten-point calibration curve was calculated using malondialdehyde standards with concentrations from 0 to 0.1 mM of malondialdehyde (dimethylacetal; MDA, Merck, Switzerland). Absorbance was read at 532 nm and results were expressed in mM MDA mg^{-1} protein.

Total protein levels were determined using the Bradford assay (Bradford Bradford, 1976) to normalize enzyme activity in each sample, with all results (excluding SOD activity) being divided by the total protein content of the respective animal and tissue.

2.5. Metabolic enzyme activity measurements

Activities of citrate synthase (CS; EC 2.3.3.1) and lactate dehydrogenase (LDH; EC 1.1.1.27) were determined in the muscle of juvenile *S. aurata*, following the methods described in Rosa et al. (2016). Samples were homogenized in ice-cold conditions in 1 mL of phosphate buffered saline solution specific for each analysis: i) CS – 20 mM HEPES (Gibco, USA), 1 mM EDTA (Triplex, Merk, Switzerland), Triton 1 % (Sigma Aldrich, Germany), pH 7.40 ± 0.02; ii) LDH – 150 mM Imidazole (Carl Roth, Germany), 1 mM EDTA (Triplex), Triton 1 %, pH 7.40 ± 0.02. Homogenates were centrifuged in 1.5 mL microtubes at 10,000 × g for 10 min (4 °C) and the supernatant was transferred to new microtubes and frozen (−80 °C) until further analysis. Each sample was analysed in triplicate in 96-well microplates. CS activity determination was based on the reaction of coenzyme A with DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid), Sigma-Aldrich, Germany). Absorbances were read at 412 nm for 5 min. LDH activity measurement was based on the oxidation of NADH and using 1 M of pyruvic acid as substrate and absorbances were read at 340 nm for 5–6 min. CS and LDH activities were expressed in U mg^{-1} protein.

2.6. Digestive enzyme activities

For digestive enzyme activity determination, the entire fish digestive tract was homogenized in 1 mL of homogenization buffer for digestive enzymes (1 mM Tris-HCl, 0.1 mM EDTA, 0.1 % Triton, pH = 7.80 ± 0.02). The crude homogenates were centrifuged in 1.5 mL microtubes at 14,000 × g for 30 min (4 °C) and the supernatant was transferred to new microtubes and frozen (−80 °C) until further analysis. Assays were adapted to 96-well microplates and were carried out using a microplate reader (Thermo Scientific Multiskan GO, USA). Each sample was analysed in triplicate.

The amylase (EC 3.2.1.1) activity in fish intestines was determined using the method described in Zaharudin et al. (2018), and starch as substrate. The absorbance was measured at 540 nm and results were expressed in mU mg^{-1} protein.

Pepsin (EC 3.4.23.1) activity was determined according to the methods described by Worthington (1982), Comabella et al. (2006), and Anson (1938), using haemoglobin 2.5 % at pH 2 as the substrate. Absorbances were immediately read at 280 nm. Pepsin activity results were presented in μU mg^{-1} protein.

Trypsin (EC 3.4.21.4) activity was determined based on the procedures described by Erlanger et al. (1961), and Klomklao et al. (2006), using BAPNA (Nα-benzoyl-DL-arginine 4-nitroanilide; Sigma Aldrich, Germany) as substrate. Absorbances were read at 405 nm immediately (T0) and again after 20 min (T20). Results were expressed in mU mg^{-1} protein.

Lipase (EC 3.1.1.3) activity was measured using lipase activity assay kit (MAK046, Sigma-Aldrich, Germany) according to the manufacturer protocol. Absorbances were read at 570 nm after 2–3 min (T0) and again after incubating the reaction at 37 °C for 73 min (T73). Results were expressed in μU mg^{-1} protein.

2.7. Statistical analysis

To evaluate significant differences in animal fitness indexes, feed efficiency, biochemical biomarkers and digestive enzyme activities between dietary treatments, two-way ANOVAs were carried out, using treatment (CTR, LD-1.5 %, LD-3 % and LD-6 %) and sampling time (T30 and T60) as variables, after assessing normality by Kolmogorov-Smirnov test and homoscedasticity by Levene's test. Data were square-rooted whenever one of the ANOVA assumptions was not verified. Post-hoc Tukey HSD tests were conducted for multiple comparisons when significant differences were found at a significance level of 0.05. All statistical analyses were performed using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

3.1. Growth performance and feed efficiency

Growth performance and fitness indexes of gilthead seabream juveniles supplemented with L. *digitata* are presented in Table 2. Although higher TL and W were found compared to the initial baseline values, no significant differences were observed between treatments and between the sampling days ($p > 0.05$). When compared to the control group, there were no significant effects on K in both sampling days due to the supplementation of seaweed ($p > 0.05$). However, upon 60 days of feeding trial, a significantly lower HSI was found in fish fed with the aquafeed containing 6 % of L. *digitata* ($p = 0.011$). Comparing sampling days (T30 and T60), significantly higher HSI was observed in the control treatment and in the dietary treatments with 1.5 % and 3 % of *L. digitata* after 60 days of feeding trial ($p = 0.007$, $p = 0.004$ and $p = 0.001$, respectively). Fish fed with 1.5 % of *L. digitata* revealed significantly higher SGR compared to the control treatment at T30 ($p = 0.042$). The feed conversion rate (FCR) was not significantly affected by the dietary treatments in any of the sampling days ($p > 0.05$). The two-way ANOVA *p*-values for the interactions between treatment (CTR, LD-1.5 %, LD-3 % and LD-6 %) and sampling time (T30 and T60) for each parameter evaluated are available in the Supplementary Materials (Table S1).

3.2. Antioxidant status indicators and lipid peroxidation

The results of the antioxidant enzymes activities (CAT, GST and SOD) and lipid peroxidation (LPO) in fish tissues are shown in Figs. 1 and 2, respectively (two-way ANOVA *p*-values for the interactions between treatment and sampling time for each biomarker evaluated can be consulted in the Supplementary Materials; Table S1).

Overall, distinct tendencies were observed according to tissue and sampling days. In fish muscle, CAT activity was affected by the addition

Table 2

Weight (W), total length (TL), Fulton's condition index (K), hepatosomatic index (HSI), specific growth rate (SGR) and feed conversion ratio (FCR) in *S. aurata* after 30 (T30) and 60 (T60) days of feeding trial (mean ± SD, $n = 12$).

Dietary treatment		W (g)	TL (cm)	K	HSI (%)	SGR (% day^{-1})	FCR
T30	CTR	12.40 ± 1.82	9.50 ± 0.32	1.44 ± 0.11	1.59 ± 0.18*	1.71 ± 0.25^{b}	0.92 ± 0.20
	LD-1.5 %	15.35 ± 2.22	10.25 ± 0.42	1.42 ± 0.12	1.62 ± 0.29*	2.37 ± 0.36^{a*}	0.81 ± 0.16
	LD-3 %	13.49 ± 2.53	9.67 ± 0.67	1.48 ± 0.05	1.69 ± 0.20*	1.78 ± 0.35^{b}	0.88 ± 0.16
	LD-6 %	14.43 ± 1.85	10.08 ± 0.55	1.40 ± 0.06	1.66 ± 0.12	1.70 ± 0.26^{b}	0.92 ± 0.15
T60	CTR	15.90 ± 1.50	10.22 ± 0.27	1.49 ± 0.11	2.23 ± 0.12$^{a\#}$	1.61 ± 0.17	1.04 ± 0.14
	LD-1.5 %	13.03 ± 3.39	9.52 ± 0.71	1.48 ± 0.09	2.28 ± 0.28$^{a\#}$	1.29 ± 0.43$^{\#}$	1.27 ± 0.49
	LD-3 %	16.78 ± 2.06	10.37 ± 0.51	1.50 ± 0.09	2.46 ± 0.24$^{a\#}$	1.63 ± 0.06	0.83 ± 0.04
	LD-6 %	17.40 ± 2.16	10.73 ± 0.55	1.40 ± 0.07	1.81 ± 0.21^{b}	1.88 ± 0.10	0.68 ± 0.05

In each column, different letters denote significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (* or #) indicate significant differences between sampling days (T30 and T60) for the same treatment. The absence of letters or symbols indicates no statistical difference. Abbreviations: CTR – control feed; LD-1.5 % – 1.5 % inclusion of L. *digitata*; LD-3 % – 3 % inclusion of *L. digitata*; LD-6 % – 6 % inclusion of *L. digitata*.

of L. *digitata* in both sampling days (Fig. 1A). In T30, fish exposed to the LD-1.5 % and LD-6 % diet revealed lower CAT activity levels compared to fish fed with the control diet ($p < 0.001$). In T60, a significant decrease was observed in fish fed the seaweed-supplemented diets ($p < 0.05$), with lower levels of CAT activity being observed in fish fed the 1.5 % inclusion level of L. *digitata* ($p < 0.001$). Comparing the treatments between sampling days, lower CAT activity was found in fish from LD-1.5 % and LD-3 % treatments after 60 days of trial ($p = 0.002$ and $p = 0.003$, respectively). GST activity levels in the muscle tissue decreased in all treatments after 60 days ($p < 0.05$; Fig. 1B). Moreover, significantly higher GST activity was found in all treatments in T60 compared with T30 ($p < 0.05$; Fig. 1B). Concerning SOD, higher activity was observed in fish fed with a diet with 3 % supplementation with L. *digitata* in T30 ($p = 0.032$; Fig. 1C). Conversely, SOD activity significantly decreased in fish fed the LD-3 % diet after 60 days of trial ($p = 0.008$; Fig. 1C). Comparing sampling days, lower SOD activity was found LD-3 % in T60 ($p < 0.001$; Fig. 1C). Lipid peroxidation (LPO) levels were significantly lower than the control for the 1.5 % and 3 % seaweed-supplementation treatments ($p < 0.001$) in T30, while after 60 days of trial lower LPO was observed in all seaweed-supplementation treatments ($p < 0.05$) and particularly in fish fed with LD-1.5 % diet, where the lowest value was recorded ($p = 0.002$; Fig. 2A). As for differences between sampling days, lower LPO was found in all treatments after 60 days ($p < 0.05$; Fig. 2A).

In fish gut, the supplementation of diets with 6 % of *L. digitata* induced the activity of CAT after 30 days of feeding trial ($p < 0.001$; Fig. 1D). After 60 days, CAT activity levels were significantly higher in fish fed with LD-3 % ($p < 0.001$) and LD-6 % ($p < 0.001$), however, a significant decrease was observed in fish fed the LD-1.5 % diet ($p = 0.001$; Fig. 1D). GST activity was also induced by the supplementation with 6 % of *L. digitata* in T30 ($p < 0.001$), whereas a significant decrease was observed in fish fed with 1.5 % supplemented diet after 60 days of trial ($p < 0.001$; Fig. 1E). Overall, higher levels of GST activity were observed in T60 in relation to T30 ($p < 0.05$; Fig. 1E). In *S. aurata* gut, lipid peroxidation was affected by the different dietary treatments in both sampling days, and the lowest LPO values were recorded in fish fed the LD-1.5 % and LD-3 % diets in T30 ($p < 0.001$; Fig. 2B) and in fish fed the LD-1.5 % diet in T60 ($p < 0.001$: Fig. 2B).

In the liver, the supplementation of L. *digitata* in fish diets decreased the activity of CAT in all the inclusion levels tested after 30 days of trial ($p < 0.05$; Fig. 1G). After 60 days, lower CAT activity levels were observed in fish fed the LD-3 % diet compared to control ($p = 0.030$; Fig. 1G). Between sampling days, lower CAT activity was observed in all treatments, including control, in T60 ($p < 0.05$; Fig. 1G). GST activity decreased in all supplemented treatments in relation to the control and lowest values of activity were found in fish fed the LD-1.5 % and LD-3 % diets in T30 ($p < 0.001$; Fig. 1H), however, after 60 days, GST activity did not significantly vary among treatments ($p > 0.05$). Significant differences between sampling days were observed in the control and LD-6 % treatments, with lower GST activity levels observed in fish sampled in T60 ($p < 0.05$; Fig. 1H). SOD liver activity was not significantly affected by the addition of macroalgae in T30 ($p > 0.05$), while in T60 a significant decrease was found in LD-3 % treatment in relation to the control treatment ($p = 0.017$; Fig. 1I). Lastly, the 1.5 % level of L. *digitata* inclusion significantly decreased lipid peroxidation in fish liver in both sampling days in relation to control fish ($p = 0.002$ and $p = 0.003$, respectively; Fig. 2C). Overall, lower levels of LPO were found after 60 days of trial compared with T30 LPO values ($p < 0.05$), except for LD-1.5 % treatments ($p > 0.05$: Fig. 2C).

3.3. Metabolic enzyme activities

Fig. 3 presents the activities of metabolic enzymes (citrate synthase and lactate dehydrogenase) in fish muscle (two-way ANOVA *p*-values for the interactions between treatment and sampling time for each biomarker evaluated can be consulted in the Supplementary Materials; Table S1).

A significant decrease in CS activity levels was observed between the control group and LD-6 % treatment at T30 ($p = 0.022$; Fig. 3A) and in LD-1.5 %, LD-3 % and LD-6 % after 60 days of trial ($p = 0.001$, $p = 0.0152$ and $p = 0.001$, respectively; Fig. 3A). LDH activity was also affected by the different dietary treatments in both sampling days. In T30, LDH muscle activity increased in fish exposed LD-6 % treatment ($p < 0.001$; Fig. 3B), while at T60 a significant increase was found between control fish and fish fed the LD-1.5 % diet and a significant decrease was observed in fish fed the LD-3 % diet ($p = 0.022$ and $p = 0.011$, respectively; Fig. 3B).

3.4. Digestive enzyme activities

The effect of the dietary inclusion of L. *digitata* on the digestive enzyme activities (trypsin, amylase, pepsin and lipase) is presented in Table 3 (two-way ANOVA *p*-values for the interactions between treatment and sampling time for each biomarker evaluated are available in the Supplementary Materials; Table S1).

. Amylase activity was higher in fish sampled after 30 days and fed with 1.5 % of *L. digitata* inclusion level in relation to the control treatment ($p = 0.029$). However, no significant differences were found between the control and the macroalgae-supplementation treatments in T60 ($p > 0.05$). Comparing dietary treatments between sampling days, higher amylase activity was observed in all treatments in T60, including control ($p < 0.05$). Pepsin activity significantly decreased in fish fed the 3 % of *L. digitata* (equivalent to a 51.1 % decrease; $p = 0.029$) at T30, while at T60 significantly lower activity was observed in fish fed the LD-3 % (26.4 % decrease; $p = 0.005$) and LD-6 % diets (34.1 % decrease; $p = 0.001$), compared with the control treatment. Comparing sampling days, higher pepsin activity levels were observed in all treatments, including the control treatment, in T60 ($p < 0.05$). Trypsin activity was significantly different among the dietary treatments at T60. Fish fed the 3 % of *L. digitata* inclusion level diet demonstrated the highest level of trypsin activity (corresponding to a 158.5 % increase in relation to the control treatment; $p < 0.001$), followed by 6 % (78.3 % increase; $p = 0.014$) and 1.5 % (58 % increase; $p = 0.006$) comparing to fish fed the

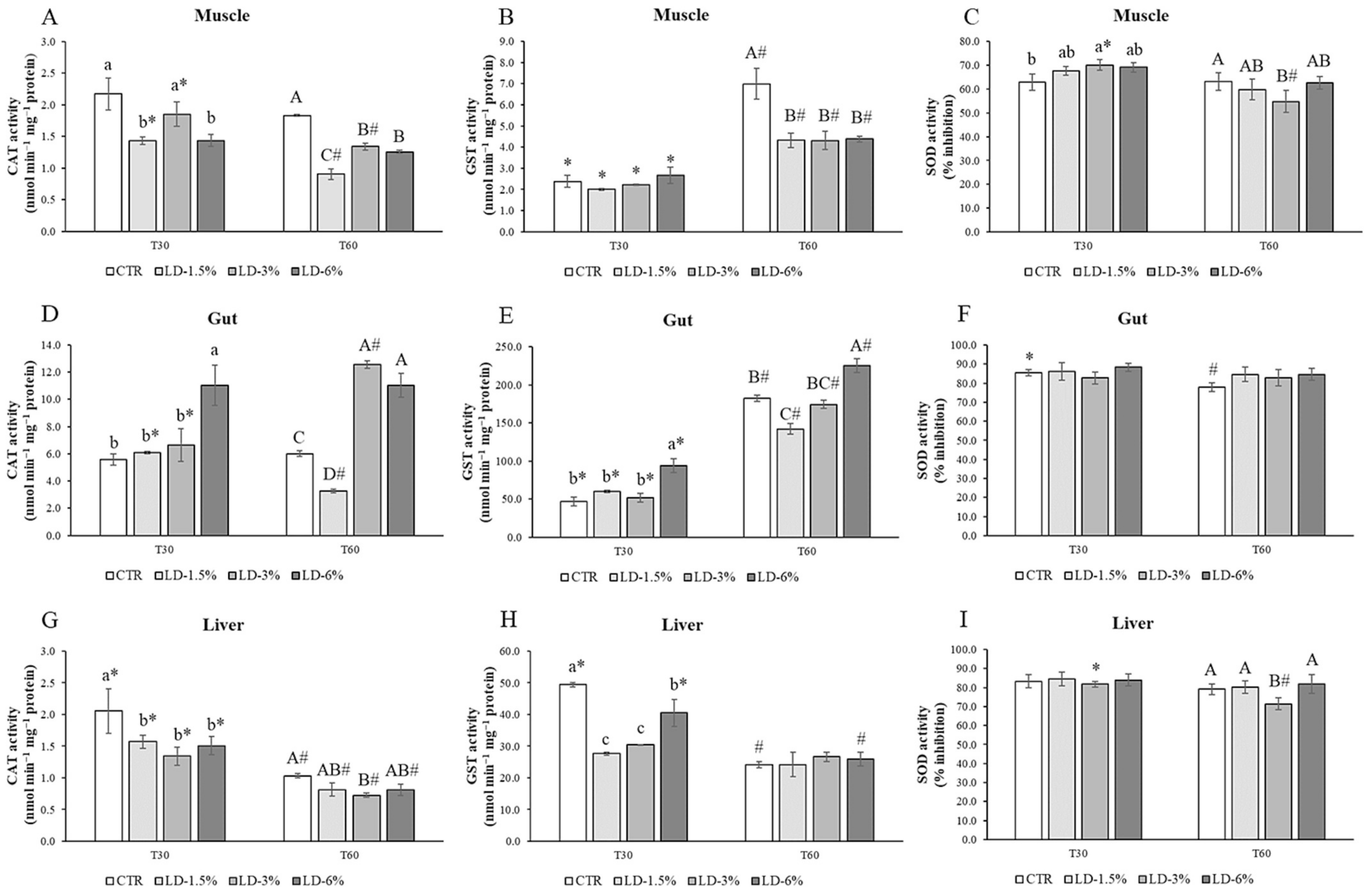

Fig. 1. Antioxidant enzyme activity (CAT, nmol min^{-1} mg^{-1} protein; GST, nmol min^{-1} mg^{-1} protein; SOD, % inhibition) in muscle (A, B, C), gut (D, E, F) and liver (G, H, I) after 30 (T30) and 60 (T60) days of feeding trial (mean ± SD, $n = 6$). Different letters denote significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (* or #) indicate significant differences between sampling days (T30 and T60) for the same treatment. The absence of letters or symbols indicates no statistical difference. Abbreviations: CTR – control feed; LD-1.5 % – 1.5 % inclusion of L. *digitata*; LD-3 % – 3 % inclusion of *L. digitata*; LD-6 % – 6 % inclusion of *L. digitata*; CAT – catalase; GST – glutathione S-transferase; SOD – superoxide dismutase.

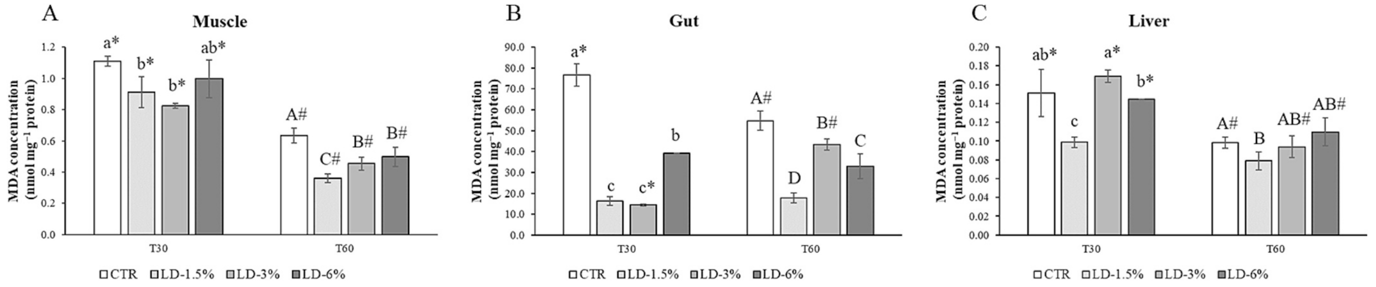

Fig. 2. Lipid peroxidation (nmol mg^{-1} protein) in muscle (A), gut (B) and liver (C) after 30 (T30) and 60 (T60) days of feeding trial (mean ± SD, $n = 6$). Different letters denote significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (* or #) indicate significant differences between sampling days (T30 and T60) for the same treatment. The absence of letters or symbols indicates no statistical difference. Abbreviations: CTR – control feed; LD-1.5 % – 1.5 % inclusion of *L. digitata*; LD-3 % – 3 % inclusion of *L. digitata*; LD-6 % – 6 % inclusion of *L. digitata*.

control diet. No significant differences were found in trypsin activity among treatments at T30 ($p > 0.05$). Overall, comparing the same treatment between sampling days, significantly higher trypsin activity was observed in LD-1.5 %, LD-3 % and LD-6 % in T60 ($p = 0.032$, $p < 0.001$ and $p < 0.001$, respectively). Regarding lipase activity no significant differences were observed between the control and the seaweed-supplementation treatments at both T30 and T60 ($p > 0.05$).

4. Discussion

Although brown seaweeds have relatively low protein content, they continue to be favoured as potential additives in animal feed due to their abundance in bioactive compounds (Gupta and Abu-Channam, 2011). However, they have received less attention in terms of application in aquafeeds when compared to other classes of seaweeds (Wan et al., 2018). This study assessed the viability of incorporating the brown macroalgae L. *digitata* as an alternative nutrient source to enhance juvenile *S. aurata* growth performance, antioxidant status and digestive function.

The findings of this study reflect the overall effects of an innovative and alternative diet supplemented with a sustainably produced ingredient with functional and nutritional attributes, i.e. dried L. *digitata*.

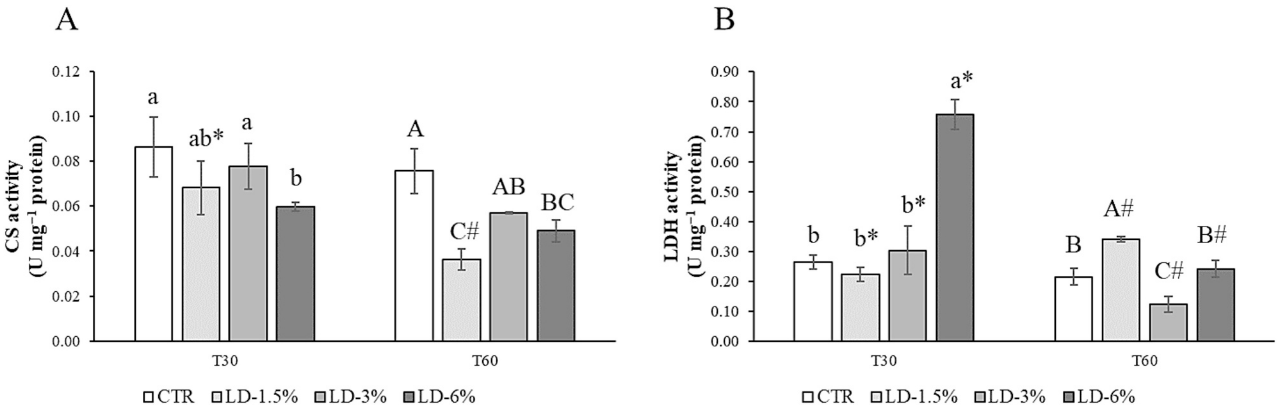

Fig. 3. A - Citrate synthase activity (U mg^{-1} protein) and B - lactate dehydrogenase activity (U mg^{-1} protein) in muscle after 30 (T30) and 60 (T60) days of feeding trial (mean ± SD, $n = 6$). Different letters denote significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (* or #) indicate significant differences between sampling days (T30 and T60) for the same treatment. The absence of letters or symbols indicates no statistical difference. Abbreviations: CTR – control feed; LD-1.5 % – 1.5 % inclusion of *L. digitata*; LD-3 % – 3 % inclusion of *L. digitata*; LD-6 % – 6 % inclusion of *L. digitata*; CS – citrate synthase; LDH – lactate dehydrogenase.

Table 3
Digestive enzyme activities in the digestive tract of *S. aurata* fed the experimental diets for 30 (T30) and 60 (T60) days (mean ± SD, n = 6).

Dietary treatment		Amylase (mU mg^{-1} protein)	Pepsin (μU mg^{-1} protein)	Trypsin (mU mg^{-1} protein)	Lipase (μU mg^{-1} protein)
T30	CTR	25.97 ± 8.46^{b*}	338.91 ± 24.93^{a*}	39.11 ± 10.35^{ab}	27.48 ± 4.03^{ab}
	LD-1.5 %	45.71 ± 7.99^{a*}	342.37 ± 50.46^{a*}	53.81 ± 12.32^{a*}	43.46 ± 13.96^{a}
	LD-3 %	34.74 ± 10.29^{ab*}	165.75 ± 32.69^{b*}	28.10 ± 3.33^{b*}	23.02 ± 7.00^{ab}
	LD-6 %	30.54 ± 9.61^{ab*}	425.14 ± 77.75^{a*}	37.51 ± 15.27^{ab*}	20.70 ± 7.44^{b}
T60	CTR	93.82 ± 24.85$^{\#}$	880.31 ± 91.43 $^{A\#}$	46.81 ± 6.57^{C}	53.59 ± 21.92
	LD-1.5 %	124.85 ± 33.84$^{\#}$	788.80 ± 173.92$^{AB\#}$	74.12 ± 7.59$^{B\#}$	42.52 ± 16.30
	LD-3 %	113.70 ± 13.15$^{\#}$	647.57 ± 118.00$^{BC\#}$	121.40 ± 17.03 $^{A\#}$	42.71 ± 9.69
	LD-6 %	126.14 ± 18.76$^{\#}$	579.95 ± 7.20$^{C\#}$	82.67 ± 2.52$^{B\#}$	32.45 ± 5.07

0.5In each column, different letters denote significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (* or #) indicate significant differences between sampling days (T30 and T60) for the same treatment. The absence of letters or symbols indicates no statistical difference. Abbreviations: CTR – control feed; LD-1.5 % – 1.5 % inclusion of L. *digitata*; LD-3 % – 3 % inclusion of *L. digitata*; LD-6 % – 6 % inclusion of *L. digitata*.

Such supplementation required, on the other hand, a proportional reduction of a plant ingredient (wheat) in order to ensure the diet's isoproteic and isolipidic balance (see diets composition in Table 1). Hence, the present findings should be interpreted as an outcome of an innovative diet formulation as whole, and not of dried L. *digitata* individually.

In the literature, the effects of seaweed supplementation on fish growth performance are inconsistent and dependent on macroalgae species and dose of inclusion, as well as, on the selected biological model (Norambuena et al., 2015; Guerreiro et al., 2019). In the current study, despite the absence of significant differences in fish morphometry (W and TL) and condition (K), a significant improvement of SGR was observed in animals fed with 1.5 % of *L. digitata*, pointing out to a potential beneficial effect on fish performance at the lowest inclusion level upon 30 days of supplementation. Ribeiro et al. (2015) reported that the inclusion of 10 % *L. digitata* in *S. aurata* diets did not influence growth performance and feed conversion. However, it is important to note that the previous study had a longer trial duration of 118 days compared to the current study. Kamunde et al. (2019) found higher SGR in Atlantic salmon (*Salmo salar)*, also a marine carnivorous species, fed with 3 % and 10 % brown seaweed flakes (AquaArom) prepared with *Laminaria* sp. (kelp) supplemented diets during a 30-day feeding trial. The results of the current study revealed that the highest inclusion level of L. *digitata* (6 %) resulted in lower hepatosomatic index (HSI) after 60 days of feeding. Similar results have been reported in *S. aurata* juveniles fed diets with 5 % inclusion level of *Gracilaria cornea* and *Ulva rigida* over a 70-day period (Vizcaíno et al., 2015). This reduction only observed in fish exposed to the highest inclusion percentage during the longest period could be due to the presence of bioactive compounds in L. *digitata* that may become deleterious after a certain dose and feeding duration, hampering gilthead seabream hepatic function (e.g., liver's enzymatic activity, ability to metabolize compounds and/or lipid deposition), or even causing apoptosis in this tissue (Kime, 1998).

In this way, the results of this study, coupled with previous studies, suggest a significant reliance on factors such as dosage, fish species and the specific type of algae used in aquafeed formulations. This emphasizes the importance of characterizing macroalgae in terms of nutritional content and biological activity. Furthermore, it underscores the relevance of studies aiming to optimize specific doses of macroalgae for a particular fish species. Additionally, it should be emphasized that the present findings are limited to the selected rearing conditions used in this experimental design, and therefore should be further validated in future studies addressing the effects of other relevant variables such as stocking densities, different amount/rates of feeding and animal life stages, among others.

SOD along with CAT are the primary antioxidant enzymes for radical scavenging, playing an important role in cells' protective mechanisms against oxidative stress, which is a chain reaction process characterized by the repetitive formation of oxygen reactive species (ROS) (Halliwell, 2006). The accumulation of ROS molecules may result in lipid peroxidation (LPO), i.e., the oxidation of fatty acids in cell membranes forming lipid peroxides (Repetto et al., 2012). SOD is responsible for the conversion of superoxide radicals into hydrogen peroxide and molecular oxygen, whereas CAT converts hydrogen peroxide into oxygen and water (Halliwell and Gutteridge, 1985). The activities of SOD and CAT are associated with the condition of the organism, which is influenced by the nutritional status, environmental stress, among other factors (Winston and Di Giulio, 1991) Elevated activity of these antioxidant enzymes typically suggests a greater presence of radicals that need to be scavenged (Andersen et al., 1998; Ross et al., 2001; Chien et al., 2003). In addition to SOD and CAT, GST is considered a major second-phase detoxification enzyme (Sheehan et al., 2001), playing a role in preventing oxidative damage by generating less toxic and more hydrophilic compounds through the conjugation of breakdown products of lipid peroxidase to glutathione (GSH; Barata et al., 2005; Park et al., 2017). Overall, in muscle and liver tissues, a decrease in the activity of antioxidant enzymes (CAT, GST and SOD) was observed in fish supplemented with L. *digitata*, especially in LD-1.5 %, suggesting a favourable maintenance of the redox state or, in other words, a reduced need to scavenge ROS and lipid peroxides in these tissues. Moreover, in line with the decreased CAT, GST and SOD activities, fish fed with lower doses of L. *digitata*, in particular at 1.5 % inclusion, showed a significant reduction in the production of lipid peroxides (LPO) in both tissues and mainly at the end of the trial. These findings are in line with a previous study where the inclusion of *Gracilaria pygmaea,* a red macroalga, in rainbow trout diet reduced SOD activity and lipid peroxidation in the liver (Sotoudeh and Mardani, 2018). Seaweeds are known to be rich in bioactive compounds (secondary metabolites), making them a potential source of nutraceuticals for animal feed supplementation (Costa et al., 2021). Brown algae, such as L. *digitata*, generally have higher antioxidant activity compared with red and green algae (Generalić Mekinić et al., 2021), mainly due to their content of phlorotannins (phenolic compounds), which are involved in the protection against oxidative damage (Galindo et al., 2023). In this regard, it is plausible that the antioxidant compounds found in L. *digitata* prevent the accumulation of oxidative compounds, like lipid peroxidation products, in muscle and liver tissues, thus diminishing the antioxidant enzyme activity requirements.

In fish gut, a distinct pattern was observed in the activity of antioxidative stress enzymes. In general, the highest L. *digitata* supplementation levels (3 % e 6 %) induced CAT, GST and SOD activities, indicating that these diets led to a certain level of oxidative stress in the gut of gilthead seabream. These differences could be attributed to the distinct antioxidant machinery strategies that are tissue-related, as well as to the fact that the gastrointestinal tract is the first organ in direct contact with nutrients and bioactive compounds released from feeds upon digestion, therefore, being particularly reactive to changes in dietary habits. Indeed, the intestine is recognized as a tissue characterized by high cell turnover, making it more susceptible to oxidative status and, therefore, seems to require high activity of antioxidant enzymes (Castro et al., 2015). The increased activity of antioxidant enzymes in the gut might be linked with the necessity to counteract the elevated oxidation potential resulting from diets with higher L. *digitata* concentrations. As previously stated, another potential explanation for this antioxidant activity enhancement might be the presence of compounds (like toxic metals, e.g., arsenic; Ribeiro et al., 2017; Barbosa et al., 2022) with the ability to induce oxidative stress through cycling between oxidation states of metals (such as As, Fe, etc.) or by interacting with antioxidants, leading to the accumulation of free radicals (Halliwell and Whiteman, 2004). However, fish fed with L. *digitata* evidenced significantly lower LPO values, regardless of seaweed dose, therefore, showing that this antioxidant scavenging activity enhancement in fish gut was able to successfully prevent tissue damage. Lastly, it is also worth mentioning that CAT and GST activities exhibited the lowest levels in fish supplemented with the lowest dose of L. *digitata* (1.5 %) for 60 days, suggesting that the minimal inclusion level was the most efficient in terms of antioxidant response modulation.

The metabolic performance of gilthead seabream supplemented with L. *digitata* was evaluated through the measurement of citrate synthase and lactate dehydrogenase activities in muscle tissue. Changes in the metabolic machinery may be due to adaptations to a particular pathway and the extent to which that pathway is employed (Pörtner, 2012; Strobel et al., 2013), and these alterations can gradually result in a shift in the energy production process (Rosa et al., 2016). Estimating the activity of key metabolic enzymes, e.g., CS and LDH, representatives of both the aerobic and anaerobic pathways, provides relevant information on animal physiological status, stress response and efficiency in attaining from nutrients the energy that is crucial to undertake vital biological activities (Rosa et al., 2016). In the present study, fish fed with 6 % of *L. digitata* showed, overall, lower CS activity coupled with a significant increase in LDH activity, suggesting a preference for the anaerobic pathway at this level of L. *digitata* supplementation, which is usually a reflex of increased stress conditions that lead to lower oxygen levels (Torres et al., 2012). Under stressful conditions, the anaerobic pathway becomes active, leading to an increased concentration of lactate in animal tissues (Kumar et al., 2010). Despite elevated LDH activity is usually associated with hampered metabolism and nutrient conversion efficiency, in this study, growth performance remained unaffected which is a positive outcome. Nevertheless, it should be noted that after 60 days of feeding trial, a significant reduction of CS activity and enhancement of LDH activity was noted in all supplemented treatments suggesting that the use of L. *digitata* for extended periods should be avoided, regardless of dose.

To date and to the best of the authors' awareness, studies that investigated the impact of macroalgae supplementation on activities of metabolic enzymes in marine fish are still very limited. This scarcity of research hinders the interpretation and comparison of the results. Therefore, it becomes essential for future research to evaluate the consistency of the findings obtained in this study under comparable sampling conditions, enabling a deeper interpretation of L. *digitata* beneficial or detrimental effects in marine fish physiology. Additionally, complementary investigation, such as aerobic scope studies, could enhance the comprehensiveness of the results.

Understanding how different dietary ingredients influence the digestive performance of farmed fish is a crucial element for optimizing aquafeed formulations (Deguara et al., 2003). Intestinal enzymes are correlated to fish nutritional status, and the activities of pancreatic secretion enzymes, such as trypsin, amylase and lipase, can serve as indicators of the digestive capacity of farmed fish (Engrola et al., 2007; Vizcaíno et al., 2014). In this study, the inclusion of L. *digitata* in fish diets did not cause a decrease in trypsin, amylase and lipase activities at any of the inclusion levels tested. In fact, a significant increase of trypsin activity was observed in fish fed the L. *digitata* supplemented diets, particularly in those fed with 3 % inclusion level diet after 60 days of trial. This suggests a potential beneficial impact on the hydrolysis of dietary proteins. Moreover, a significant increase in amylase activity was observed in fish fed diets enriched with 1.5 % inclusion level of L. *digitata* after 30 days of trial*,* compared with fish fed the control diet. Amylase activity can be negatively affected by the dietary level of starch (Kokou et al., 2016). Carnivorous fish, such as *S. aurata*, are expected to have a reduced ability to digest starch, compared to herbivorous and omnivorous species (Enes et al., 2011; Liang et al., 2022). Nevertheless, in the present study, diets with higher L. *digitata* concentrations (3 % and 6 % inclusion level) did not impact amylase activity even as the starch content decreased from 11.50 % to 7.71 % due to alterations in wheat meal content, and regardless of the feeding trial duration. These findings concerning amylase activity suggest that *S. aurata* juveniles may be able to adapt to variations in their dietary habits to a certain extent. Regarding lipase activity results, it is important to acknowledge that the Sigma-Aldrich assay used does not include bile salts, which are essential for the activity of bile-salt dependent lipase (BSDL), the primary digestive lipase in teleost fish (Nolasco-Soria, 2023). Consequently, the assay's limitations may have influenced the observed lipase activity results and may not fully reflect the functional role of BSDL in fish digestive process. Yet, a different tendency was observed in pepsin activity. Pepsin, as essential digestive enzyme, is prominently involved in protein digestion in fish feeds (Mazumder et al., 2018). In the present study, lower pepsin activity was observed in fish fed with LD-3 % diet after 30 days of trial and with LD-3 % and LD-6 % after 60 days of trial. Horie et al. (1995) suggested that dietary fiber would inhibit pepsin activity and consequently fish growth performance since the initial stages of digestion occur in the stomach of carnivorous fish (Greenwood, 1953). The results obtained in this study are supported by the previous hypothesis, as the fiber content in the experimental diets increased with the greater percentage of L. *digitata* inclusion*,* resulting in reduced pepsin activity in fish fed diets with higher macroalgae concentrations (i.e., 3 % and 6 % inclusion levels). Nevertheless, despite the decrease in pepsin activity, the overall growth performance of fish remained similar. Apart from digestive enzyme activities, the structure and morphology of the intestinal mucosa plays a very important role in nutrient absorption (Vizcaíno et al., 2014). The impact of seaweed on intestinal histomorphology may be influenced by various factors, including fish species, the type of seaweed used in the diet, the method of feed processing and the inclusion of other ingredients in the diets (Guerreiro et al., 2019). Consequently, it would be valuable for further research to investigate the impact of incorporating L. *digitata* in diets on the intestinal function and morphology of farmed gilthead seabream.

5. Conclusions

The present study confirmed that L. *digitata* constitutes a promising alternative feed ingredient with interesting nutritional and functional attributes that may be used in aquafeeds for carnivorous fish, such as juvenile *S. aurata*, maintaining or even improving animal growth performance and overall well-being (depending on the dose). The decrease in tissues' oxidative stress pointed out an enhanced antioxidant capacity and ability to prevent the formation of tissue-damaging free radicals, particularly in fish supplemented with 1.5 % of *L. digitata*.

Considering the less positive outcomes observed in fish supplemented with the highest dose of L. *digitata* (6 %) and/or for the longest period (60 days), i.e., enhancement of antioxidant enzyme activities and LDH, and decreased CS and pepsin activities, lower doses (<3 %) and short periods of supplementation (30 days) should be preferred whenever using this seaweed as an ingredient in marine fish feed formulas.

Even though the present results constitute a relevant body of evidence on L. *digitata* beneficial effects on farmed marine fish performance and welfare, further research endeavours (accounting for the occurrence of stress challenges, e.g. disease outbreak and/or sub-optimal rearing conditions, and tackling complementary endpoints, e.g. animal aerobic scope, immunity, nutrient digestibility and intestinal functionality), are still required prior to the establishment of the most cost-effective seaweed supplementation dose and the subsequent validation of this strategy at the industrial level.

Ethical statement

Fish trials were performed in accordance with legal European regulations (EU Directive 2010/63) and were previously approved by IPMA's Animal Welfare and Ethics Body (ORBEA), overseen by the Portuguese National Competence Authority (Direção-Geral de Alimentação e Veterinária, DGAV). Fish handling, sampling and maintenance were performed by certified researchers by the Federation of European Laboratory Animal Science Associations (FELASA).

CRediT authorship contribution statement

Alícia Pereira: Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation. **Isa Marmelo:** Writing – review & editing, Methodology, Investigation. **Marta Dias:** Writing – review & editing, Methodology. **Patrícia Anacleto:** Writing – review & editing, Methodology. **Carla Pires:** Writing – review & editing, Methodology. **Irineu Batista:** Writing – review & editing, Methodology. **António Marques:** Writing – review & editing, Validation, Supervision, Resources. **Ana Luísa Maulvault:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741984.

Data availability

Data will be made available on request.

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