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Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (*Dicentrarchus labrax*)

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ABSTRACT

This work aimed to appraise growth performance, digestive capacity and immune and stress responsiveness in European seabass (*Dicentrarchus labrax*) when fed diets supplemented with seaweeds. A control diet (without supplementation) was tested against 6 practical diets supplemented either with *Gracilaria* spp., *Ulva* spp., or *Fucus* spp., at 2.5 or 7.5% levels, plus an additional diet with a blend of the three seaweeds, each supplemented at 2.5% (*Mix*). Seabass juveniles (24.0 ± 6.3 g) were fed the experimental diets for 84 days. Dietary seaweed supplementation had no effect on growth rate (DGI: 1.0–1.1), voluntary feed intake (11.3–12.6 g kg $^{-1}$ ABW^{-1} day^{-1}), feed conversion ratio (FCR: 1.2–1.4) and protein efficiency ratio (PER: 1.5–1.8). Lipase activity was significantly higher in fish fed *Mix* diet when compared to control ($P < 0.05$). Glutathione peroxidase (GPx) was significantly affected by seaweeds presence regardless its level of supplementation ($P < 0.05$). The innate immune system was significantly altered by dietary seaweed supplementation ($P < 0.05$). Fish fed the 7.5% seaweed supplemented diets had a significant decrease in the ACH50 level, when compared to fish fed 2.5% seaweed supplemented diets ($P < 0.05$). Moreover, a combined effect of seaweed and supplementation level significantly affected lysozyme (LYS) activity ($P < 0.05$). Fish fed diet with *Ulva* at 2.5% had an increase in LYS when compared to fish fed control and *Ulva* at 7.5% diets. Overall, our results indicate that the use of dietary seaweed supplementation improves immune and antioxidant responses in European seabass without compromising growth performance.

1. Introduction

Seaweeds are considered a vast source of biologically active substances, especially rich in essential nutrients for human and animal nutrition (Jiménez-Escrig et al., 2011). Under *in vitro* conditions, relevant antimicrobial and anti-viral activities (Hemmingson et al., 2006; Cox et al., 2010; Narasimhan et al., 2013), and efficient antioxidant capacity (Leonard et al., 2011; Narasimhan et al., 2013), have been widely demonstrated in extracts obtained from representative species of Chlorophyta, Rhodophyta and Phaeophyta. *In vivo* studies carried out in swine, demonstrated that the dietary supplementation with *Laminaria* sp. (*Phaeophyta*) extracts, containing laminarin and fucoidan (polysaccharides), improved gastrointestinal health and growth performance of starter and weaned pigs (Reilly et al., 2008; Leonard et al., 2011). In ruminants subjected to pre-slaughter stress conditions, the dietary supplementation with brown seaweed (*Ascophyllum nodosum*) increased the antioxidant status by lowering lipid peroxidation (LPO), increasing glutathione peroxidase activity, and reducing stress indicators (Kannan et al., 2007; Archer, 2005). In crustaceans, extracts and sulphated galactans from red seaweeds (*Gracilaria fisheri* and *Asparagopsis* spp.) administered to black tiger shrimp (*Penaeous monodon*) also caused a significant immune-stimulant

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effect and an increased protection against the pathogen *Vibrio* spp. and white spot syndrome virus (Kanjana et al., 2011; Wongprasert et al., 2014; Rudtanatip et al., 2015; Manilal et al., 2012). In fish, the administration of diets with a binder produced from *A. nodosum* caused an increase in lysozyme levels of Atlantic salmon (*Salmo salar*) (Gabrielsen and Austreng, 1998).

Nevertheless, dietary seaweed incorporation in aquafeeds for finfish has shown contradictory growth results. El-Tawil (2010) found higher growth performance in Red tilapia (*Oreochromis* sp.) fed diets up to 15% level of *Ulva* sp. supplementation. Mustafa et al. (1995) tested the inclusion of 5% seaweed supplementation with *Ascophyllum* sp., *Porphyra* sp. and *Ulva* sp., in Red seabream (*Pagrus major*). These authors found enhanced growth in fish fed all three supplemented diets and evidenced *Porphyra* sp. as showing the most pronounced growth. *Porphyra* sp. supplementation in Rainbow trout (*Oncorhynchus mykiss*) also showed enhanced growth up to 10% level of supplementation (Soler-Vila et al., 2009). In contrast, 9 and 18% *Porphyra* sp. inclusion in thick-lipped grey mullet (*Chelon labrosus*) showed impaired growth when compared with non-supplemented diets (Davies et al., 1997).

In vitro studies have reported increased respiratory burst and immune system stimulation, through rapid release of reactive oxygen species (ROS) and signaling proteins, by *Ulva rigida* and *Chondrus crispus* extracts and β-glucans in turbot (*Scophthalmus maximus*) and Atlantic salmon (*S. salar*) phagocytes (Dalmo and Seljelid, 1995; Castro et al., 2004). Besides immunocompetency, a positive correlation has been reported between the phenolic content and antioxidant capacity of seaweeds (Devi et al., 2011), using strategies as ROS scavenging activity or lipid peroxidation inhibition (Heo et al., 2005). Orange-spotted grouper (*Epinephelus coicoides*), previously inoculated with sodium alginate from *Macrocystis pyritera* and carrageenan from *C. crispus* showed an increase in respiratory burst, superoxide dismutase (SOD) and phagocytic activities after exposition to *Vibrio alginolyticus* (Cheng et al., 2007). Recent results observed in Nile tilapia (*Oreochromis mossambicus*) have demonstrated that the administration of extracts and products from red (*Gracilaria folifera*) and brown (*Padina gymnospora* and *Sargassum cinereum*) seaweeds may be effective as therapeutic and prophylactic treatments against *Pseudomonas* spp. infection (Thanigaivel et al., 2015a,b). Additional research efforts are undoubtedly required to carry out *in vivo* studies, which are scarce in fish, to accurately evaluate the potential of seaweed application as a nutritional tool (Makkar et al., 2015). The utilization of immunostimulant functional ingredients, such as meals, extracts and isolated compounds, may unfold as particularly appropriate in aquaculture. Practical studies assessing if dietary seaweed incorporation contributes to enhance the basal health status of cultivated fish are highly recommended, since stocking and harvesting routine practices such as crowding, size sorting and transport, constitute usual stressors in fish farming.

The objective of this study was to examine the effect of dietary seaweed supplementation on growth performance, digestive capacity, and immune and stress responsiveness, using seabass (*Dicentrarchus labrax*) as an experimental model. Specifically, we tested the hypothesis that seaweeds could be used to enhance immune and antioxidant defenses in fish, without growth impairments.

2. Material and methods

The current study was conducted under the supervision of accredited researchers in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes of the European directive 2010/63/UE.

2.1. Seaweeds and experimental diets

Seaweed from 3 distinct phyla, *Gracilaria* spp. (Rhodophyta), *Ulva* spp. (Chlorophyta) and *Fucus* spp. (Phaeophyta) were cultivated in an integrated multi-trophic aquaculture (IMTA) system and supplied by ALGAPLUS Lda. (Portugal). The 3 seaweeds were supplemented separately to the experimental diets at two levels, 2.5% and 7.5% dry matter basis (DM). Two additional diets were formulated, one control diet without seaweed supplementation (*Ctrl*) and the other (*Mix*) with all three seaweeds, each at 2.5% level of supplementation, dry matter basis (DM). Proximate and mineral compositions of the seaweeds are depicted in Table 1.

The 8 experimental diets were formulated to be isoenergetic (22 kJ g^{-1} DM), isoproteic (47% DM) and isolipidic (18% DM) (Table 2). All diets were formulated with the same practical ingredients. Marine-derived ingredients (fishmeal and fish oil) represented 36.5% DM of the diet, whereas dietary protein and fat levels were chosen in accordance with recommendations for juvenile seabass (Webster and Lim, 2002; FAO, 2005–2015). All diets were supplemented with crystalline indispensable amino acids (L-Lysine and DL-Methionine) to meet this species requirement. The dietary macronutrient balance after seaweed incorporation was achieved by adjusting the soy protein concentrate and wheat meal contents. All ingredients were finely grounded (hammer mill, 0.8 mm sieve), mixed and then extruded (twin screw extruder, 2.0 mm pellet size, SPAROS, Portugal). Diets were finally dried at 45 °C for 12 h and stored at 4 °C until used.

2.2. Fish and rearing conditions

The growth trial was conducted at the Aquatic Engineering laboratory of Instituto de Ciências Biomédicas Abel Salazar (ICBAS, Porto, Portugal). Seabass juveniles were supplied by a commercial fish farm (MARESA, Spain). After arrival at the experimental unit, fish were kept under quarantine conditions for a two-week period. Thereafter, fish were individually weighed (initial body weight: 24.0 ± 6.3 g) and distributed to the 24 units of 80 L tanks (3 tanks/treatment; 17 fish/tank) connected to a closed recirculation seawater system. The water temperature was maintained at 20 ± 1 °C. Total ammonium (≤ 0.5 mg L^{-1}), nitrate (≤ 3 mg L^{-1}) and pH levels (≈8) were measured during the entire trial to ensure levels within the recommended ranges for marine species. A photoperiod was set for 12 h light:12 h dark and the flow rate in each tank set for 90 L/h. Fish were hand-fed to apparent satiety twice a day (10.00 and 16.00 h) for 84 days.

At the end of the feeding trial, fish were fasted for 24 h, prior to sampling. Fish were anesthetized with 100 mg L^{-1} of MS-222 (Sigma-Aldrich Co., LLC, Bellfonte, USA) and individually weighed (g). Thereafter, fish were sacrificed by decapitation and body tissues were collected. Liver and viscera were weighted from 4 fish per tank to calculate the hepatosomatic (HIS) and viscerosomatic (VSI) indexes. Other 4 fish from each tank were used for whole body composition analyses. Additionally, 6 fish were sampled from each tank for the analyses to be carried out in the intestine, blood and liver. Plasma was obtained by blood centrifugation (5 min, 10000 rpm). All samples were stored at −80 °C until subsequent analysis.

2.3. Analytical methods

Carcasses from 4 fish from each tank were milled, pooled and the moisture content determined (105 °C for 24 h). Samples were subsequently lyophilized before further analyses. Both feed and whole body analyses were carried out in duplicate following the

Table 1
Proximate and mineral composition of the used seaweed meals.

	Limit Detection (LD)	*Gracilaria* spp.		*Fucus* spp.		*Ulva* spp.	
Mineral comp. (mg kg^{-1} dry tissue)		Mean	SD	Mean	SD	Mean	SD
Lead (Pb)	0.004	1.1	0.1	0.8	0.1	2.8	0.1
Mercury (Hg)	0.005	<LD		<LD		<LD	
Cadmium (Cd)	0.031	0.2	0.0	0.1	0.0	0.1	0.0
Tin (Sn)	0.005	0.4	0.0	0.9	0.2	0.5	0.0
Arsenic (As)	0.074	<LD		<LD		<LD	
Iron (Fe)	1.021	511.0	31.0	560.0	19.0	4080.0	246.0
Zinc (Zn)	1.908	43.0	1.0	109.0	2.0	47.0	1.0
Copper (Cu)	0.206	1.5	0.1	2.8	0.2	5.0	0.1
Selenium (Se)	0.832	6.5	0.3	8.8	0.5	9.8	0.3
Mineral comp. (%)							
Potassium (k)	0.034	13.1	0.5	11.3	0.5	9.3	2.0
Sodium (Na)	0.025	11.7	1.6	3.3	0.1	19.9	1.1
Magnesium (Mg)	0.001	0.3	0.0	0.4	0.0	2.0	0.2
Calcium (Ca)	0.002	0.2	0.0	0.9	0.1	0.4	0.0
Phosphorus (P)	0.006	0.3	0.1	0.2	0.0	0.4	0.1
Proximate composition (%DM)							
Dry matter		93.4	0.0	87.0	0.0	85.7	0.0
Crude protein		25.9	0.2	17.2	0.2	23.2	0.2
Ash		34.3	0.2	20.7	0.1	34.8	0.3
Crude fat		1.1	0.7	3.4	0.1	1.5	0.1
Gross energy (kJ g^{-1} DM)		12.8	0.2	15.1	0.0	12.1	0.1

Table 2
Feed formulation and proximate composition of the experimental diets.

	Dietary treatments							
	Ctrl	Gr2.5	Gr7.5	Ul2.5	Ul7.5	Fu2.5	F7.5	Mix 7.5
Fish ingredients (%DM)								
Fishmeal Standard	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Fishmeal SOLOR	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Soy protein concentrate (Soycomil)	11.8	11.3	10.0	11.5	10.4	11.5	11.0	10.3
Wheat gluten	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Corn gluten	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Soybean meal 48	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Rapeseed meal	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Wheat meal	9.0	7.0	3.3	6.8	2.9	6.8	2.3	3.0
Peas gelatinized (Aquatex 8071)	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Fish oil—COPPENS	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Soybean oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Rapeseed oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vit & Min Premix PV01	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Binder (Kieselghur)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant powder (Paramega)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
MCP	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Lysine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
DL-Methionine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Gracilaria sp.		2.5	7.5					2.5
Ulva sp.				2.5	7.5			2.5
Fucus sp.						2.5	7.5	2.5
Proximate composition (%DM)								
Dry matter	94.7	96.5	95.9	94.3	94.2	94.5	95.0	94.8
Ash	8.6	9.4	10.8	9.5	11.1	8.9	10.0	10.6
Crude protein	47.8	47.4	47.8	47.8	47.7	47.7	47.9	47.9
Crude fat	19.1	18.1	19.6	19.5	19.1	18.1	18.4	19.3
Gross energy (kJ g^{-1} DM)	22.7	22.8	22.4	22.7	22.3	22.9	22.7	22.4

methodology described by AOAC (2006). Ash was analyzed by combustion (500 °C during 6 h) in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany) and crude protein (N × 6.25) using a Leco N analyzer (Model FP-528, Leco Corporation, St. Joseph, USA). Crude lipid content was determined by petroleum ether extraction (40–60 °C) using a Soxtec™ 2055 Fat Extraction System (Foss, Hilleroed, Denmark) while gross energy was quantified in an adiabatic bomb calorimeter (Werke C2000 basic, IKA, Staufen, Germany).

Whole intestines were homogenized for digestive enzymes activity quantification according to the method described by Rungruangsak-Torrissen (2007). Lipase activity was determined according to Winkler and Stuckmann (1979). Trypsin and chymotrypsin were assessed through the production of nitroaniline (Rungruangsak-Torrissen and Sundby, 2000). α-amylase activity was examined by formation of the disaccharide maltose (Bernfeld, 1951). In addition, the ratios amylase to trypsin (A/T) and trypsin to chymotrypsin (T/C) were calculated to infer about preferable energy source and growth rates, respectively. According to Hidalgo et al. (1999), an elevated A/T ratio can translate into higher capacity to digest carbohydrates, sparing protein for growth. The ratio T/C has been associated with feed conversion efficiency and specific growth rate, and can indicate if fish are in a fast or slow growth phase (Rungruangsak-Torrissen et al., 2006). The protein content

(Lowry et al., 1951) of the homogenate was analyzed to establish results in terms of enzymatic units per protein unit.

Innate immune response was evaluated in plasma by determination of alternative complement (ACH50) and lysozyme activities, as well as peroxidase content. The ACH50 activity followed the protocol described by Sunyer and Tort (1995) and was defined as the reciprocal of the serum dilution capable of inducing 50% lysis of rabbit erythrocytes. Lysozyme activity was evaluated by turbidimetric assay (Ellis, 1990) of the *Micrococus lysodeikicus* lysis and later standardization by hen egg white lysozyme (Sigma, Portugal) reaction curve. Peroxidase levels were examined as the chemical reduction of 3,3′, 5,5′—tetramethylbenzidine hydrochloride (Sigma, Portugal), according to Quade and Roth (1997).

Livers were homogenized (phosphate buffer, 0.1 M pH 7.4) as a previous step to measure indicators related to oxidative status. The protein content was determined according to Bradford (1976) and used to standardize antioxidant enzymes activities. Lipid peroxidation was determined by quantifying the presence of thiobarbituric acid reactive substances (Ohkawa et al., 1979). Catalase (EC 1.11.1.6.) activity was studied based on Clairborne (1985), with hydrogen peroxide (30%) as substrate. Glutathione s-transferase (GST) (EC 2.5.1.18) was determined by absorbance at 340 nm, using 1-chloro-2,4-dinitrobenzene as substrate, consistent with the methods described in Habig et al. (1974). Glutathione peroxidase (GPx) (EC 1.11.1.9.) and Glutathione reductase (GR) (EC 1.8.1.7) were evaluated based on NADPH (Sigma, Portugal) oxidation at 340 nm (Cribb et al., 1989; Mohandas et al., 1984). Total and oxidized Glutathione were evaluated at 412 nm by the formation of 5-thio-2-nitrobenzoic acid, as detailed in Baker et al. (1990). Reduced glutathione (GSH) was calculated as the difference between total and oxidized glutathiones.

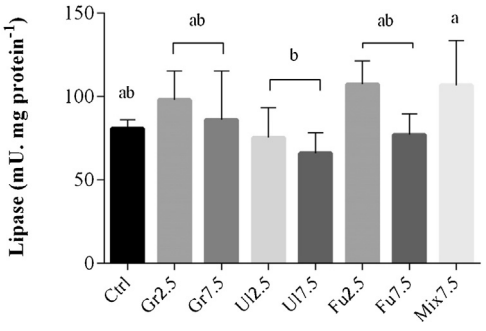

Fig. 1. Lipase activity measured in intestines of sea bass fed diets supplemented with no seaweed (*Ctrl*) or with *Gracilaria* sp. (*Gr*), *Ulva* sp. (*Ul*), and *Fucus* sp. (*Fu*) at 2.5 or 7.5% and *Mix* (*Gr* + *Ul* + *Fu*) for 84 days. Different letters indicate significant differences for the presence of seaweed, despite the level of supplementation.

2.4. Growth performance calculations

Growth performance was calculated as follows. Daily growth index (DGI) = $100 \times [(FBW)^{1/3} - (IBW)^{1/3}] \times$ trial duration in days, whereas FBW and IBW are the final and initial average body weights (g); Feed conversion ratio (FCR) = FI (g)/weight gain (g), whereas FI is feed intake on dry matter basis; protein efficiency ratio (PER) = weight gain (g)/protein intake (g). The hepatosomatic and viscerosomatic index (HSI and VSI) were calculated as: HSI = 100 × [liver weight (g)/fish weight (g)]; VSI = 100 × [viscera weight (g)/fish weight (g)].

2.5. Statistical analyses

All data were checked for normality (Shapiro–Wilk test) and homogeneity of variances (Leveneís test). The analysis of variance was performed applying two-way ANOVA test, with seaweed (SW)

Table 3
Growth performance, feed utilization and feed intake of sea bass fed the experimental period for 84 days. Corresponding P values to seaweed (S) and concentration (C) factors, as same as interaction S × C, are exhibited for every parameter.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S × C
Growth	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Initial Body Weight (g)	23.5	1.2	23.6	1.2	24.5	1.2	24.5	0.9	23.2	1.1	24.3	1.7	24.4	1.2	23.9	1.9	0.502	0.679	0.971
Final Body Weight (g)	51.7	3.5	55.5	3.9	58.2	4.3	54.8	0.8	53.9	5.7	55.3	1.3	51.9	3.6	53.1	6.1	0.554	0.217	0.953
DGI	1.0	0.1	1.1	0.1	1.2	0.1	1.1	0.0	1.1	0.1	1.1	0.1	1.0	0.1	1.1	0.1	0.270	0.215	0.868
FCR	1.4	0.1	1.3	0.1	1.2	0.1	1.3	0.0	1.3	0.2	1.2	0.1	1.4	0.2	1.3	0.1	0.380	0.29	0.841
PER	1.5	0.1	1.7	0.1	1.8	0.1	1.7	0.0	1.6	0.2	1.7	0.1	1.5	0.2	1.6	0.2	0.319	0.290	0.869
Intake (g kg ABW^{-1} day $^{-1}$)																			
DM	12.6	0.4	12.0	0.6	11.5	0.4	11.6	0.3	12.3	0.8	11.3	0.6	11.7	0.7	11.7	0.8	0.213	0.771	0.696

N = 3 tanks. Absence of letters indicates non-significant differences between treatments ($P \geq 0.05$) for every studied factor and subsequent interaction.
DGI, Daily growth index = $100 \times ((\text{Final body weight})^{1/3} - (\text{Initial body weight})^{1/3})$/days; FCR, Feed conversion ratio = dry feed intake/weight gain; PER, Protein efficiency ratio = weight gain/crude protein intake; ABW, Average body weight; DM, Dry matter.

Table 4
Somatic index and whole body composition of sea bass fed the experimental diets for 84 days. Corresponding P values to seaweed (S) and concentration (C) factors, as same as interaction S × C, are exhibited for every parameter.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S x C
Somatic index (%)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
HSI	1.2	0.1	1.5	0.2	1.5	0.0	1.4	0.1	1.5	0.1	1.4	0.1	1.4	0.1	1.5	0.3	0.675	0.437	0.651
VSI	7.9	0.4	8.2	0.4	8.4	0.7	8.6	0.6	8.6	0.3	8.1	0.6	8.2	0.4	8.0	1.1	0.852	0.730	0.567
Whole body composition (%WW)																			
DM	31.4	0.3	32.3	3.6	31.3	0.5	32.0	1.1	31.2	0.7	31.8	1.9	31.9	1.4	31.8	3.2	0.987	0.791	0.777
Ash	4.8	0.1	4.1	0.4	4.4	0.8	4.3	0.6	4.3	0.7	4.9	0.7	4.7	0.2	4.5	0.5	0.645	0.212	0.943
Protein	18.0	0.3	18.0	1.6	17.7	0.7	17.9	0.6	16.9	1.0	17.8	1.1	18.1	1.0	17.7	0.6	0.780	0.531	0.405
Fat	9.7	0.5	10.6	2.1	9.4	0.6	10.3	0.4	9.7	0.9	9.2	1.4	9.1	0.5	9.8	2.7	0.755	0.269	0.794
Energy (kJ g^{-1} WW)	7.8	0.3	8.1	1.2	7.6	0.1	8.1	0.2	7.5	0.1	7.7	0.8	7.6	0.5	7.7	1.7	0.957	0.360	0.654

N = 3 tanks. Absence of letters indicates non-significant differences between treatments ($P \geq 0.05$) for every studied factor and subsequent interaction.
HSI, Hepatosomatic index; VSI, Viscerosomatic index; WW, Wet weight.

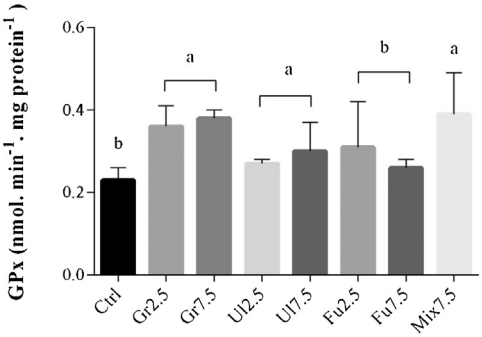

Fig. 2. Glutathione peroxidase (GPx) activity measured in liver of sea bass fed diets supplemented with no seaweed (*Ctrl*) or with *Gracilaria* sp. (*Gr*), *Ulva* sp. (*Ul*), *Fucus* sp. (*Fu*) at 2.5 or 7.5% and *Mix* (*Gr*+*Ul*+*Fu*) for 84 days. Different letters indicate significant differences for the presence of seaweed, despite the level of supplementation.

(*Gr*, *Ul*, *Fu* and *Mix*) and concentration (C) (2.5 and 7.5%) as independent factors, with an un-supplemented diet as control treatment. Significant differences were considered when $P < 0.05$. The statistical software package used was SPSS Statistics 21 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Growth performance, feed intake and whole body composition

Fish mortality was lower than 2% in all experimental treatments. All dietary treatments exhibited a 2.2 fold increase in their initial body weights (24 ± 6.3 g) after 12 weeks of feeding trial. No significant differences were observed on growth (DGI range of 1.0–1.1), VFI (11.3–12.6 g kg ABW^{-1} day^{-1}), FCR (1.2–1.4) and PER (1.5–1.8), among dietary treatments (Table 3). Both HIS, VFI and body composition did not vary among the dietary treatments ($P > 0.05$) (Table 4). Especially relevant was the fact that whole body composition, evaluated in terms of macronutrients and energy, also showed no significant differences between dietary treatments (Table 4).

3.2. Digestive enzyme activities

Amylase (1.0–1.6 U. mg protein^{-1}), chymotrypsin (4.1–6.5 U. mg protein^{-1}), trypsin (10.0–14.8 U. mg protein^{-1}) activities and the ratios amylase/trypsin (111.0–221.6) and trypsin/chymotrypsin (2.0–3.5) are reported in Table 5. Digestive enzymes activities were not significantly different among the dietary treatments ($P > 0.05$). Fish fed *Ulva* spp. had the lower lipase activity (Fig. 1) among all treatments, significantly different from fish fed *Mix* diet ($P < 0.05$). An interaction between seaweed type and incorporation level was not observed ($P > 0.05$).

3.3. Antioxidant status indicators

Seabass juveniles fed the experimental diets exhibited similar ($P > 0.05$) levels of hepatic catalase (CAT) and lipid peroxidation (LPO) as presented in Table 6.

Glutathione s-transferase (GST), total glutathione (GT), glutathione reductase (GR), oxidized glutathione (GSSG) and reduced glutathione (GSH) are shown in Table 7. Both seaweed type and supplementation level revealed no significant differences for GST, GT, GR, GSSG and GSH ($P > 0.05$), in contrast, glutathione peroxidase (GPx) activity (Fig. 2) was significantly higher in fish fed *Gracilaria* spp., *Ulva* spp. and *Mix*, when compared to the other dietary treatments ($P < 0.05$).

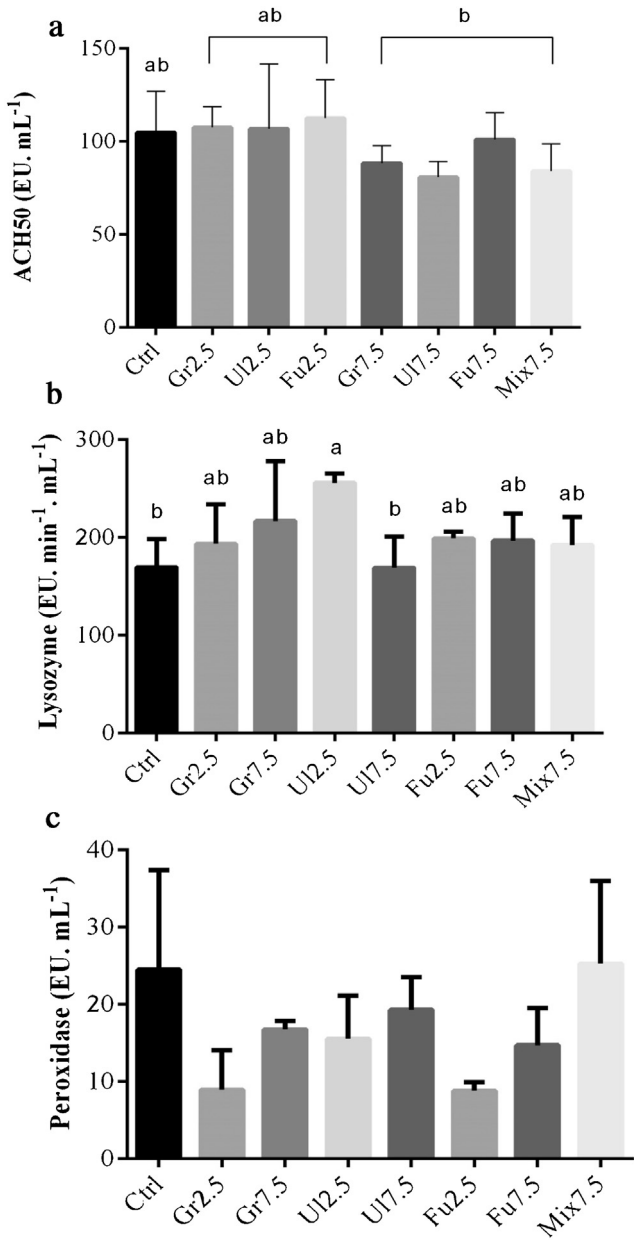

Fig. 3. Alternative complement pathway (a), lysozyme activity (b) and peroxidase level (c) determined in plasma of sea bass fed diets supplemented with no seaweed (*Ctrl*) or with *Gracilaria* sp. (*Gr*), *Ulva* sp. (*Ul*), *Fucus* sp. (*Fu*) at 2.5 or 7.5% and *Mix* (*Gr*+*Ul*+*Fu*) for 84 days. In Fig. 3 (a), different letters are an indication of statistical differences between levels of supplementation. In Fig. 3 (b), significant differences were found for the interaction of factors presence of the seaweed and level of supplementation.

3.4. Innate immune parameters

Fish fed dietary seaweed supplementation at 7.5% DM incorporation level showed a significant decrease in alternative complement pathway (ACH50) when compared to the other dietary treatments ($P > 0.05$) (Fig. 3a). A combined effect caused by both seaweed type and level of supplementation was observed on lysozyme activity in the case of diets containing *Ulva* spp. (Fig. 3b). Fish fed *Ul2.5* diet showed a significantly higher lysozyme activity (255.6 EU. min^{-1} mL^{1}) in comparison with those fed the *Ctrl* (169.4 EU min^{-1} mL^{-1}) and *Ul7.5* diets (168.9 EU min^{-1} mL^{-1}). Plasma peroxidase levels (Fig. 3c) showed no significant differences ($P > 0.05$) between dietary treatments.

Table 5
Digestive enzyme activities in intestines of sea bass fed the experimental diets for 84 days.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S × C
Digestive enzyme activity	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Amylase (U mg protein^{-1})	1.2	0.1	1.5	0.3	1.4	0.4	1.6	0.0	1.4	0.3	1.0	0.1	1.5	0.1	1.3	0.2	0.179	0.100	0.416
Chymotrypsin (U mg protein^{-1})	4.4	2.1	4.1	1.4	4.2	0.6	4.2	1.4	5.5	2.0	5.6	0.6	6.5	3.2	4.2	1.6	0.569	0.065	0.880
Trypsin (U mg protein^{-1})	14.8	4.5	12.5	6.3	10.0	5.5	13.5	7.2	11.4	6.3	12.8	5.8	10.0	1.3	11.3	11.2	0.999	0.853	0.698
A/T	119.7	50.5	156.5	92.9	195.6	52.4	221.6	116.4	172.3	106.0	111.0	68.4	199.0	25.0	150.6	75.2	0.625	0.424	0.550
T/C	3.5	1.7	3.0	1.0	2.4	1.2	2.9	0.6	2.2	1.0	2.5	0.6	2.0	0.6	2.8	1.6	0.890	0.317	0.707

N = 6 intestines/treatment. Values presented as Mean ± Standard Deviation.
Absence of letters indicates non-significant differences between treatments ($P \geq 0.05$) for every studied factor and subsequent interaction.
A, Amylase; T, Trypsin; C, Chymotrypsin.

Table 6
Catalase activity and lipid peroxidation determined in the liver of sea bass fed the experimental diets for 84 days.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S × C
Antioxidant indicators	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Catalase (μmol min^{-1} mg protein^{-1})	89.5	14.2	86.8	7.8	89.8	6.2	90.2	6.2	91.5	4.9	98.5	3.9	81.1	17.7	86.7	7.8	0.471	0.761	0.278
LPO (nmol TBARS g tissue^{-1})	41.5	28.5	43.2	10.6	59.3	20.3	52.6	8.0	22.7	2.6	36.8	26.6	44.1	28.2	32.7	18.2	0.528	0.089	0.809

N = 6 livers/treatment. Values presented as Mean ± Standard Deviation.
Absence of letters indicates non-significant differences between treatments ($P \geq 0.05$) for every studied factor and subsequent interaction.
LPO, Lipid Peroxidation.

Table 7
Antioxidant enzyme activities (nmol min^{-1} mg protein^{-1}) measured in liver of fish fed the experimental diets for 84 days.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S × C
Antioxidant enzymes	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
GST	44.5	13.4	50.1	5.9	51.9	1.8	51.3	8.2	52.0	11.6	60.8	13.3	53.5	17.7	54.2	7.9	0.855	0.416	0.826
TG	8.3	2.4	15.8	3.7	13.2	3.5	12.5	3.9	8.3	1.6	16.5	4.6	9.8	2.4	13.6	7.4	0.381	0.244	0.097
GR	56.1	3.3	56.0	6.4	31.9	9.6	56.2	10.0	52.5	5.0	50.1	14.9	45.0	16.2	45.0	10.4	0.176	0.812	0.066
GSSG	4.8	0.7	5.3	0.8	3.2	0.3	5.1	1.5	3.4	0.5	5.0	0.5	3.1	0.3	6.1	3.9	0.240	0.353	0.092
GSH	5.03	0.9	10.6	4.4	9.98	3.2	7.40	2.4	5.61	1.2	12.5	5.5	6.69	2.3	7.52	3.6	0.219	0.372	0.223

N = 6 livers/treatment. Values presented as Mean ± Standard Deviation.
Absence of letters indicates non-significant differences between treatments ($P \geq 0.05$) for every studied factor and subsequent interaction.
GST, Glutathione s-transferase; TG, Total Glutathione; GR, Glutathione Reductase; GSSG, Oxidized Glutathione; GSH, Reduced Glutathione.

4. Discussion

In the present work, the dietary supplementation of *Gracilaria* spp., *Ulva* spp., and *Fucus* spp., at 2.5 and 7.5% levels, in diets for European seabass had no effect on final body weight, DGI, FCR and PER (Table 3). Similar results have been described by Bagni et al. (2005) for seabass when fed diets supplemented with immunostimulants. These authors associated the lack of growth enhancements to the water temperature (22 °C), which was below the optimal for this specie. The results from growth performance and feed utilization in this work are, nonetheless, in agreement with previous data reported for seabass juveniles fed to satiety with diets containing similar protein (47%) and lipid (16–18%) levels (Guroy et al., 2006; Bonaldo et al., 2010). Previous studies showed that dietary supplementation of *Gracilaria* spp. and *Ulva* spp. were an adequate protein source at 10% inclusion level (Valente et al., 2006). Similarly, Queiroz et al. (2014) and Linares et al. (2014) working with gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*), respectively, did not observe changes on growth performance when fish were fed diets supplemented with seaweed. Marinho et al. (2013) established a 10% maximal level of *Ulva* spp. supplementation for Nile tilapia (*Oreochromis niloticus*). In contrast, Silva et al. (2015) and Valente et al. (2015) working with rainbow trout (*O. mykiss*) and Nile tilapia (*O. niloticus*), established a 5% inclusion of *Gracilaria* spp. as the recommended dietary supplementation level. The growth performance analyzed in this work for seabass fed the experimental diets for 84 days (12 weeks) revealed a 2.2 fold increase for all dietary treatments. With the exception of Linares et al. (2014) when working with Senegalese sole, a specie characterized by slow growth rates. All the above mentioned and discussed studies had a duration of less than 12 weeks. This enables the comparison of results and allows inferring on the adequacy of the tested diets and duration of the feeding trial for European seabass.

Amylase, trypsin and chymotrypsin (Table 5) revealed no influence from the dietary treatments. Our results are within the reported range of marine species, despite the high variability reported for alkaline enzymes activities (Hidalgo et al., 1999; Frouël et al., 2008). Lipase activity (Fig. 1) showed a decrease in the *Ul2.5* and *Ul7.5* treatments. In literature, lipid source and level of inclusion in diets are the most reported modulators of lipase activity (Infante and Cahu, 1999). The observed negative effect of *Ulva* spp. over lipase activity is in accordance with the reported lipase inhibiting proprieties found in Chlorophyta seaweeds (Bitou et al., 1999). Moreover, Francis et al. (2001) reported the presence of antinutrients in seaweeds, particularly lectins (polyphenolic functional group) that interfere with digestion and feed utilization processes. This eventual antinutritional effect associated with *Ulva* spp., is not so severe that causes growth impairment and is apparently compensated by the presence of *Gracilaria* spp. and *Fucus* spp. in *Mix* diet. The inclusion of *Gracilaria* spp. and *Ulva* spp. up to 10% DM in seabass diets has been previously tested by Valente et al. (2006)

who found no negative consequences on the apparent digestibility coefficient. To our knowledge, digestive enzymes activities were not previously studied in *D. labrax* as a response to seaweed supplemented diets.

Glutathione peroxidase (GPx) catalyzes the reduction hydrogen peroxide and lipid peroxides and is considered an efficient protective enzyme against lipid peroxidation (Zhang et al., 2004). In this study, GPx revealed significant differences between dietary treatments. Results showed higher GPx activity in groups fed *Gr2.5*, *Gr7.5* and *Mix* diets when compared with all other treatments. The similar activity observed for *Gr2.5* and *Gr7.5* diets suggests a lack of dose-response effect. Moreover, the higher GPx level in *Mix* diet supports the hypothesis that a 2.5% *Gracilaria* spp. inclusion level is sufficient to promote GPx activity. Red seaweeds as *Gracilaria* spp. contain high levels of antioxidants and represent a good source of selenium (Devi et al., 2011) which functions as GPx cofactor (Rotruck et al., 1973). In this sense it is plausible that *Gracilaria* spp. influence over GPx activity was due to a selenium increment. This result has been reported in other studies with *D. labrax* (Martínez-Páramo et al., 2014). Cell detoxification from the harmful action caused by reactive oxygen species (ROS) is the primary function of the antioxidant systems (Urso and Clarkson, 2003). The accumulation of ROS molecules may naturally arise from cellular metabolism via mitochondrial respiratory chain (Fulle et al., 2004). This accumulation leads to an oxidative imbalance (Ben Ameur et al., 2012) that may result in cell membrane lipid peroxidation (LPO) (Lesser, 2006; Ran et al., 2007). In the present study, the similarities found for LPO between dietary treatments (Table 6) and the majority of detoxifying enzymes (Table 7) further supports the evidence that dietary seaweed supplementation causes no nutritional stress to European seabass. On the other hand, depending on conditions, antioxidants can act as pro-oxidants (Herbert, 1996) and the prolonged exposure to supplemented diets could lead to a decrease in antioxidant enzymes activities. This decrease has been observed in rainbow trout after long-term exposure to propiconazole (Li et al., 2010). Overall, extensive *in vitro* work has been done on seaweed antioxidant and immune-stimulating properties using isolated compounds (Vadlapudi and Kaladhar, 2012) and experiments tackling the potential effects of dietary seaweed supplementation on the oxidative stress condition and innate immune system in fish are still scarce.

The role of the complement system in fish immunity is associated with chemotaxis, opsonization and pathogen destruction functions (Holland and Lambris, 2002). Several compounds such as glucans, tocopherol and ascorbic acid are recognized as potential complement activity enhancers (Bagni et al., 2000). Previous works have stated the presence of such compounds in seaweeds (Leiro et al., 2007; Bobadilla et al., 2013; Kanimozhi et al., 2013). In the present work, the alternative complement pathway results (Fig. 3a) were modulated by the level of seaweed supplementation. A decrease in this parameter was detected when seabass were fed diets with 7.5% DM supplementation level. ACH50 modulating factors are well established, and include stress conditions, temperature, nutritional deficiencies and additives (Boshra et al., 2006; Montero et al., 1998). Furthermore, ACH50 activity is interpreted by numerous authors as a sign of a more prompt innate immune system, improving the resistance to pathogens (Chiu et al., 2008; Biller-Takahashi et al., 2012). In our study however the 7.5% supplementation appears to jeopardize seabass immune condition. These results are similar to Araújo et al. (2015) who reported a decrease in ACH50 in rainbow trout fed 10% *Gracilaria* spp. supplemented diet. The reason may rely on the antioxidant import carried by seaweed inclusion. Ortuno et al. (2000) showed that an excessive inclusion of Vitamine E reduced ACH50 activity in gilthead seabream (*S. aurata*), suggesting also that an unbalanced dose of antioxidants may be the cause for ACH50 decrease. As mentioned before, seaweed represent a good source of antioxidants, and the 7.5% DM may represent an unbalancing point for the correct dose of antioxidants in seabass diet.

Dietary seaweed supplementation also increased lysozyme activity levels. Seabass fed the *Ul2.5* diet showed an enhanced lysozyme activity when compared to the *Ctrl* and the *Ul7.5* diet, suggesting a dose-dependent response in this immune parameter. The remaining seaweed supplemented diets showed lysozyme results similar to *Ctrl* diet. Lysozyme levels can vary considerably between different fish species and in most cases is positively correlated with disease resistance (Fevolden et al., 1994). Similarly to ACH50, lysozyme has vast literature regarding its importance as broad-spectrum enzyme with strong action against Gram-negative bacteria (Yano, 1996) and its modulation factors, both physical (Valero et al., 2014) and nutritional (Cecchini et al., 2000). Valente et al. (2015) showed *Ulva* spp. dietary inclusion of 5 and 10% DM levels to be indifferent in Nile tilapia (*O. niloticus*). Nevertheless, a lysozyme increase was found for olive flounder (*Paralichthys olivaceus*) fed increasing levels of kelp (*Ecklonia cava*) (Kim and Lee, 2008), and flathead grey mullet (*Mugil cephalus*) fed *Sargassum* spp. supplemented diets and tested against *Pseudomonas fluorescence* (Kanimozhi et al., 2013). The actual immunostimulants compounds in seaweed supplements are unknown however some studies suggest that polysaccharides present in seaweeds may activate the non-specific immune responses in both teleost and shrimps (Kim and Lee, 2008). This is in accordance with known lysozyme molecular triggers, as pathogenic microorganisms often possess polysaccharides, and other characteristic components that are not normally on the surface of multicellular organisms (Uribe et al., 2011). Fish immune response to dietary seaweed supplementation has been acknowledged as dose- and species-dependent (Araújo et al., 2015). Increased peroxidase levels have been detected in rainbow trout fed diets with 5% *Gracilaria* sp. supplementation (Araújo et al., 2015). In contradiction, no alteration in this parameter has been observed in Nile tilapia fed diets supplemented with *Ulva* sp. at 5 and 10% levels (Valente et al., 2015) which is consistent with the absence of differences in plasma peroxidase levels in our work. Another explanation for the absence of differences between dietary groups may be related with the high deviation detected in peroxidase levels. The combined results of these immunologic indicators point to an immuno-stimulating effect of seaweeds supplementation.

Further research should address seabass response to biotic or abiotic stressors, clarifying the objective role of dietary seaweed supplementation as immune and antioxidant stimulating.

5. Conclusions

Seaweed supplementation (*Gracilaria* spp., *Ulva* spp., and *Fucus* spp.) in practical diets for European seabass juveniles have no impact on growth performance, up to 7.5% supplementation level. A minimal modulation of digestive enzyme activities was found in diets with *Ulva* spp. supplementation. Dietary *Gracilaria* spp. supplementation led to an antioxidant capacity enhancement that, however, should be confirmed testing other Rhodophyta species. A dietary 2.5% incorporation level improved innate immune system indicators. Overall, our results indicate that seaweed supplementation in aquafeeds may be a valuable tool to increase the immunocompetency of valuable aquaculture fish species, without compromising growth performance.

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There are no conflicts of interest in connection with the present study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aqrep.2016.03.005.

References

Araújo, M., Rema, P., Sousa-Pinto, I., Cunha, L., Peixoto, M., Pires, M., Seixas, F., Brotas, V., Beltrán, C., Valente, L.P., 2015. Dietary inclusion of IMTA-cultivated Gracilaria vermiculophylla in rainbow trout (*Oncorhynchus mykiss*) diets: effects on growth, intestinal morphology, tissue pigmentation, and immunological response. J. Appl. Phycol., 1–11.

Archer, G.S., 2005. Reducing stress in sheep by feeding the seaweed *Ascophyllum nodosum*. In: Doctoral Dissertation. Texas A&M University.

AOAC, 2006. Official Methods of Analysis of AOAC International. AOAC International, Maryland, USA.

Bagni, M., Archetti, L., Amadori, M., Marino, G., 2000. Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*). J. Vet. Med. B Infect. Dis. Vet. Public Health 47 (10), 745–751.

Bagni, M., Romano, N., Finoia, M.G., Abelli, L., Scapigliati, G., Tiscar, P.G., Sarti, M., Marino, G., 2005. Short- and long-term effects of a dietary yeast β-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). Fish Shellfish Immunol. 18 (4), 311–325.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal. Biochem. 190 (2), 360–365.

Ben Ameur, W., de Lapuente, J., El Megdiche, Y., Barhoumi, B., Trabelsi, S., Camps, L., Serret, J., Ramos-Lopez, D., Gonzalez-Linares, J., Driss, M.R., Borras, M., 2012. Oxidative stress, genotoxicity and histopathology biomarker responses in mullet (*Mugil cephalus*) and sea bass (*Dicentrarchus labrax*) liver from Bizerte Lagoon (Tunisia). Mar. Pollut. Bull. 64 (2), 241–251.

Bernfeld, P., 1951. Enzymes of starch degradation and synthesis. Adv. Enzymol. Relat. Subj. Biochem. 12, 379–428.

Biller-Takahashi, J.D., Takahashi, L.S., Marzocchi-Machado, C.M., Zanuzzo, F.S., Sabioni, R.E., 2012. Hemolytic activity of alternative complement pathway as an indicator of innate immunity in pacu (*Piaractus mesopotamicus*). R. Bras. Zootec. 41 (2), 237–241.

Bitou, N., Ninomiya, M., Tsujita, T., Okuda, H., 1999. Screening of lipase inhibitors from marine algae. Lipids 34 (5), 441–445.

Bobadilla, F., Rodriguez-Tirado, C., Imarai, M., Galotto, M.J., Andersson, R., 2013. Soluble β-1,3/1,6-glucan in seaweed from the southern hemisphere and its immunomodulatory effect. Carbohyd. Polym. 92 (1), 241–248.

Bonaldo, A., Badiani, A., Fagioli, P., Fontanillas, R., Koppe, W., Mariani, L., Gatta, P.P., 2010. The influence of different dietary energy content and feeding regimes on growth and feed utilization of European sea bass (*Dicentrarchus labrax* L.). Ital. J. Anim. Sci. 8 (2), 842–844.

Boshra, H., Li, J., Sunyer, J.O., 2006. Recent advances on the complement system of teleost fish. Fish Shellfish Immunol. 20 (2), 239–262.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254.

Castro, R., Zarra, I., Lamas, J., 2004. Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes. Aquaculture 229 (1–4), 67–78.

Cecchini, S., Terova, G., Caricato, G., Saroglia, M., 2000. Lysozyme activity in embryos and larvae of sea bass (*Dicentrarchus labrax* L.), spawned by broodstocks fed with vitamin C enriched diets. Bull Eur Ass Fish Pathol 20 (3), 120–124.

Cheng, A.-C., Tu, C.-W., Chen, Y.-Y., Nan, F.-H., Chen, J.-C., 2007. The immunostimulatory effects of sodium alginate and iota-carrageenan on orange-spotted grouper *Epinephelus coicoides* and its resistance against *Vibrio alginolyticus*. Fish Shellfish Immunol. 22 (3), 197–205.

Chiu, S.-T., Tsai, R.-T., Hsu, J.-P., Liu, C.-H., Cheng, W., 2008. Dietary sodium alginate administration to enhance the non-specific immune responses, and disease resistance of the juvenile grouper *Epinephelus fuscoguttatus*. Aquaculture 277 (1–2), 66–72.

Clairborne, A., 1985. Catalase activity. In: CRC Handbook of Methods in Oxygen Radical Research. CRC Press, Boca Raton.

Cox, S., Abu-Ghannam, N., Gupta, S., 2010. An assessment of the antioxidant and antimicrobial activity of six species of edible irish seaweeds. Int. Food Res. J. 17, 205–220.

Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of a microplate reader in an assay of glutathione reductase using 5,5'-dithiobis(2-nitrobenzoic acid). Anal. Biochem. 183 (1), 195–196.

Dalmo, R.A., Seljelid, R., 1995. The immunomodulatory effect of LPS, laminaran and sulphated laminaran [β(1,3)-D-glucan] on Atlantic salmon, *Salmo salar* L., macrophages in vitro. J. Fish Dis. 18 (2), 175–185.

Davies, S.J., Brown, M.T., Camilleri, M., 1997. Preliminary assessment of the seaweed Porphyra purpurea in artificial diets for thick-lipped grey mullet (*Chelon labrosus*). Aquaculture 152 (1–4), 249–258.

Devi, G.K., Manivannan, K., Thirumaran, G., Rajathi, F.A., Anantharaman, P., 2011. In vitro antioxidant activities of selected seaweeds from Southeast coast of India. Asian Pac. J. Trop. Dis. 4 (3), 205–211.

El-Tawil, 2010. Effects of Green Seaweeds (*Ulva* sp.) as Feed Supplements in Red Tilapia (*Oreochromis* sp.) diet on growth performance, feed utilization and body composition. J. Arabian Aquacult. Soc. 5, 179–193.

Ellis, A.E., 1990. Lysozyme assays. In: Techniques in Fish Immunology. SOS Publications, Fair Haven, New Jersey.

FAO, 2014. The State of World Fisheries and Aquaculture. 2014 edn., Rome.

Fevolden, S., Røed, K., Gjerde, B., 1994. Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon; correlations to disease resistance. Fish Shellfish Immunol. 4 (7), 507–519.

Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199 (3–4), 197–227.

Frouël, S., Le Bihan, E., Serpentini, A., Lebel, J., Koueta, N., Nicolas, J., 2008. Preliminary study of the effects of commercial *Lactobacilli* preparations on digestive metabolism of juvenile sea bass (*Dicentrarchus labrax*). J. Mol. Microbiol. Biotechnol. 14 (1–3), 100–106.

Fulle, S., Protasi, F., Di Tano, G., Pietrangelo, T., Beltramin, A., Boncompagni, S., Vecchiet, L., Fano, G., 2004. The contribution of reactive oxygen species to sarcopenia and muscle ageing. Exp. Gerontol. 39 (1), 17–24.

Gabrielsen, B.O., Austreng, E., 1998. Growth, product quality and immune status of Atlantic salmon, Salmo salar L., fed wet feed with alginate. Aquacult. Res. 29 (6), 397–401.

Guroy, D., Devecler, E., Guroy, B., Teknay, A., 2006. Influence of feeding frequency on feed intake, growth performance and nutrient utilization in european sea bass (*Dicentrarchus labrax*) fed pelleted or extruded diets. Turk. J. Vet. Anim. Sci. 30, 171–176.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139.

Hemmingson, J.A., Falshaw, R., Furneaux, R.H., Thompson, K., 2006. Structure and antiviral activity of the galactofucan sulfates extracted from *Undaria pinnatifida* (Phaeophyta). J. Appl. Phycol. 18 (2), 185–193.

Heo, S.-J., Park, E.-J., Lee, K.-W., Jeon, Y.-J., 2005. Antioxidant activities of enzymatic extracts from brown seaweeds. Bioresour. Technol. 96 (14), 1613–1623.

Herbert, V., 1996. Prooxidant effects of antioxidant vitamins. Introduction. J. Nutr. 126 (Suppl. 4), 1197s–1200s.

Hidalgo, M.C., Urea, E., Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture 170 (3–4), 267–283.

Holland, M.C.H., Lambris, J.D., 2002. The complement system in teleosts. Fish Shellfish Immunol. 12 (5), 399–420.

Jiménez-Escrig, A., Gómez-Ordóñez, E., Rupérez, P., 2011. Chapter 26 - seaweed as a source of novel nutraceuticals: sulfated polysaccharides and peptides. In: Se-Kwon, K. (Ed.), Advances in Food and Nutrition Research, vol. 64. Academic Press, pp. 325–337.

Kanimozhi, S., Krishnaveni, M., Deivasigmani, B., Rajasekar, T., Priyadarshni, P., 2013. Immunomostimulation effects of *Sargassum whitti* on *Mugil cephalus* against *Pseudomonas fluorescence*. Int. J. Curr. Microbiol. Appl. Sci. 2 (7), 93–103.

Kanjana, K., Radtanatip, T., Asuvapongpatana, S., Withyachumnarnkul, B., Wongprasert, K., 2011. Solvent extracts of the red seaweed *Gracilaria fisheri* prevent *Vibrio harveyi* infections in the black tiger shrimp *Penaeus monodon*. Fish Shellfish Immunol. 30 (1), 389–396.

Kannan, G., Saker, K.E., Terrill, T.H., Kouakou, B., Galipalli, S., Gelaye, S., 2007. Effect of seaweed extract supplementation in goats exposed to simulated preslaughter stress. Small Rumin. Res. 73 (1–3), 221–227.

Kim, S.S., Lee, K.J., 2008. Effects of dietary kelp (*Ecklonia cava*) on growth and innate immunity in juvenile olive flounder *Paralichthys olivaceus* (Temminck et Schlegel). Aquacult. Res. 39 (15), 1687–1690.

Leiro, J.M., Castro, R., Arranz, J.A., Lamas, J., 2007. Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed *Ulva rigida* C. Agardh. Int. Immunopharmacol. 7 (7), 879–888.

Leonard, S.G., Sweeney, T., Bahar, B., Lynch, B.P., O'Doherty, J.V., 2011. Effects of dietary seaweed extract supplementation in sows and post-weaned pigs on performance, intestinal morphology, intestinal microflora and immune status. Br. J. Nutr. 106 (5), 688–699.

Lesser, M.P., 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annu. Rev. Physiol. 68, 253–278.

Li, Z.-H., Zlabek, V., Li, P., Grabic, R., Velisek, J., Machova, J., Randak, T., 2010. Biochemical and physiological responses in liver and muscle of rainbow trout

after long-term exposure to propiconazole. Ecotoxicol. Environ. Saf. 73 (6), 1391–1396.
Linares, F., Peleteiro, J.B., Álvarez-Blázquez, B., Pazos, G., Valente, L.M.P., Rodriguez, J.L., 2014. Dietary Inclusion of 10% Seaweeds in Senegalese Sole (*Solea Senegalensis*) Juveniles. Aquaculture Europe, Donostia–San Sebastián, Spain.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193 (1), 265–275.
Makkar, H.P.S., Tran, G., Heuzé, V., Giger-Reverdin, S., Lessire, M., Lebas, F., Ankers, P., 2015. Seaweeds for livestock diets: a review. Anim. Feed Sci. Technol. 212, 1–17.
Manilal, A., Selvin, J., George, S., 2012. In vivo therapeutic potentiality of red seaweed, *Asparagopsis* (*Bonnemaisoniales*, *Rhodophyta*) in the treatment of *Vibriosis* in *Penaeus monodon* Fabricius. Saudi J. Biol. Sci. 19 (2), 165–175.
Marinho, G., Nunes, C., Sousa-Pinto, I., Pereira, R., Rema, P., Valente, L.P., 2013. The IMTA-cultivated *Chlorophyta Ulva* spp. as a sustainable ingredient in Nile tilapia (*Oreochromis niloticus*) diets. J. Appl. Phycol. 25 (5), 1359–1367.
Martínez-Páramo, S., Dinis, M.T., Sarasquete, C., Cabrita, E., 2014. Vitamin, Selenium and Zinc Supplemented Diets Improved Antioxidant Defences of European Seabass and Gilthead Seabream Male Breeders. Aquaculture Europe, Donostia–San Sebastián Spain.
Mohandas, J., Marshall, J.J., Duggin, G.G., Horvath, J.S., Tiller, D.J., 1984. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. possible implications in analgesic nephropathy. Biochem. Pharmacol. 33 (11), 1801–1807.
Montero, D., Tort, L., Izquierdo, M., Robaina, L., Vergara, J., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3HUFA dietary deficiencies. Fish. Physiol. Biochem. 18 (4), 399–407.
Mustafa, G., Wakamatsu, S., Takeda T-a Umino, T., Nakagawa, H., 1995. Effects of algae meal as feed additive on growth, feed efficiency, and body composition in Red Sea Bream. Fish. Sci. 61 (1), 25–28.
Narasimhan, M.K., Pavithra, S.K., Krishnan, V., Chandrasekaran, M., 2013. In vitro analysis of antioxidant, antimicrobial and antiproliferative activity of *Enteromorpha antenna*, *Enteromorpha linza* and *Gracilaria corticata* extracts. Jundishapur J. Nat. Pharm. Prod. 8 (4), 151–159.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95 (2), 351–358.
Ortuno, J., Esteban, M., Meseguer, J., 2000. High dietary intake of α-tocopherol acetate enhances the non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 10 (4), 293–307.
Quade, M.J., Roth, J.A., 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet. Immunol. Immunopathol. 58 (3–4), 239–248.
Queiroz, A.C.S., Pereira, R., Domingues, A.F., Peixoto, M.J.D., Gonçalves, J.F.M., Ozorio, R.O.A., 2014. Effect of seaweed supplementation on growth performance, immune and oxidative stress responses in gilthead seabream (*Sparus aurata*). Front. Mar. Sci., http://dx.doi.org/10.3389/conf.fmars.2014.02.00018, Conference Abstract: IMMR | International Meeting on Marine Research.
Ran, Q., Liang, H., Ikeno, Y., Qi, W., Prolla, T.A., Roberts, L.J., Wolf, N., VanRemmen, H., Richardson, A., 2007. Reduction in Glutathione Peroxidase 4 increases life span through increased sensitivity to apoptosis. J. Gerontol. A. Biol. Sci. Med. Sci. 62 (9), 932–942.
Reilly, P., O'Doherty, J.V., Pierce, K.M., Callan, J.J., O'Sullivan, J.T., Sweeney, T., 2008. The effects of seaweed extract inclusion on gut morphology, selected intestinal microbiota, nutrient digestibility, volatile fatty acid concentrations and the immune status of the weaned pig. Animal 2 (10), 1465–1473.
Rotruck, J., Pope, A., Ganther, H., Swanson, A., Hafeman, D.G., Hoekstra, W., 1973. Selenium: biochemical role as a component of glutathione peroxidase. Science 179 (4073), 588–590.
Rudtanatip, T., Withyachumnarnkul, B., Wongprasert, K., 2015. Sulfated galactans from *Gracilaria fisheri* bind to shrimp haemocyte membrane proteins and stimulate the expression of immune genes. Fish Shellfish Immunol. 47 (1), 231–238.
Rungruangsak-Torrissen, K., Sundby, A., 2000. Protease activities, plasma free amino acids and insulin at different ages of Atlantic salmon (*Salmo salar* L.) with genetically different trypsin isozymes. Fish Physiol. Biochem. 22 (4), 337–347.
Rungruangsak-Torrissen, K., Moss, R., Andresen, L.H., Berg, A., Waagbø, R., 2006. Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.). Fish Physiol. Biochem. 32 (1), 7–23.
Rungruangsak-Torrissen, K., 2007. Digestive efficiency, growth and qualities of muscle and oocyte in atlantic salmon (*Salmo salar* L.) fed on diets with krill meal as an alternative protein source. J. Food Biochem. 31 (4), 509–540.
Silva, D.M., Valente, L.M.P., Sousa-Pinto, I., Pereira, R., Pires, M.A., Seixas, F., Rema, P., 2015. Evaluation of IMTA-produced seaweeds (*Gracilaria*, *Porphyra*, and *Ulva*) as dietary ingredients in Nile tilapia, *Oreochromis niloticus* L., juveniles. Effects on growth performance and gut histology. J. Appl. Phycol. 27 (4), 1671–1680.
Soler-Vila, A., Coughlan, S., Guiry, M., Kraan, S., 2009. The red alga *Porphyra dioica* as a fish-feed ingredient for rainbow trout (*Oncorhynchus mykiss*): effects on growth, feed efficiency, and carcass composition. J. Appl. Phycol. 21 (5), 617–624.
Sunyer, J.O., Tort, L., 1995. Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. Vet. Immunol. Immunopathol. 45 (3–4), 333–345.
Thanigaivel, S., Chandrasekaran, N., Mukherjee, A., Thomas, J., 2015a. Investigation of seaweed extracts as a source of treatment against bacterial fish pathogen. Aquaculture 448, 82–86.
Thanigaivel, S., Vidhya Hindu, S., Vijayakumar, S., Mukherjee, A., Chandrasekaran, N., Thomas, J., 2015b. Differential solvent extraction of two seaweeds and their efficacy in controlling *Aeromonas salmonicida* infection in *Oreochromis mossambicus*: a novel therapeutic approach. Aquaculture 443, 56–64.
Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in teleost fish: a review. Vet. Med. 56, 486–503.
Urso, M.L., Clarkson, P.M., 2003. Oxidative stress, exercise, and antioxidant supplementation. Toxicology 189 (1–2), 41–54.
Vadlapudi, V., Kaladhar, D., 2012. Antioxidant activities of marine algae: a review. Medicinal Plants as Antioxidant Agents: Understanding Their Mechanism of Action and Therapeutic Efficacy, Kerala, India. 189–203.
Valente, L., Gouveia, A., Rema, P., Matos, J., Gomes, E., Pinto, I., 2006. Evaluation of three seaweeds *Gracilaria bursa-pastoris*, *Ulva rigida* and *Gracilaria cornea* as dietary ingredients in European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture 252 (1), 85–91.
Valente, L.P., Araújo, M., Batista, S., Peixoto, M., Sousa-Pinto, I., Brotas, V., Cunha, L., Rema, P., 2015. Carotenoid deposition, flesh quality and immunological response of Nile tilapia fed increasing levels of IMTA-cultivated Ulva spp. J. Appl. Phycol., 1–11.
Valero, Y., García-Alcázar, A., Esteban, M.A., Cuesta, A., Chaves-Pozo, E., 2014. Seasonal variations of the humoral immune parameters of European sea bass (*Dicentrarchus labrax* L.). Fish Shellfish Immunol. 39 (2), 185–187.
Webster, C.D., Lim, C., 2002. Nutrient Requirements and Feeding of Finfish for Aquaculture. CABI.
Winkler, U.K., Stuckmann, M., 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol. 138 (3), 663–670.
Wongprasert, K., Rudtanatip, T., Praiboon, J., 2014. Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp. Fish Shellfish Immunol. 36 (1), 52–60.
Yano, T., 1996. The non-specific immune system. In: Iwama, G., Nakanishi, T. (Eds.), The Fish Immune System: Organism, Pathogen and Environment. Academic Press, San Diego, pp. 105–157.
Zhang, J., Shen, H., Wang, X., Wu, J., Xue, Y., 2004. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. Chemosphere 55 (2), 167–174.