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Evaluation of *Ulva ohnoi* as functional dietary ingredient in juvenile Senegalese sole (*Solea senegalensis*): Effects on the structure and functionality of the intestinal mucosa

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ABSTRACT

This study evaluates the effects of the macroalgae *Ulva ohnoi* on growth and intestinal functionality and integrity of *Solea senegalensis* juveniles in a 90-day feeding trial. *Ulva* diet (U-5) included 5% dry *U. ohnoi* biomass, whereas an algae-free diet was used as control. After being fed for 45 and 90 days, fish body weight was recorded for growth evaluation, and biological samples were taken. The inclusion of *U. ohnoi* (5%) significantly affected growth performance of sole juveniles, given that after being fed for 45 and 90 days fish showed lower body weight, specific growth and weight gain rates than fish fed with the control diet. Compared to fish fed with control diet, protease activities measured in the pancreatic secretion was also reduced when *Ulva* was included in the diet, but these fish also showed increased alkaline phosphatase activity in their intestinal mucosa. Quantitative PCR revealed changes in the relative expression of a set of genes coding for enzymes involved in lipid absorption and metabolism, several digestive enzymes, as well as for some stress response indicators. Electron microscopy revealed a lack of damage in the intestinal mucosa, as well as significantly increased mucosal absorptive surface in those fish fed with *Ulva*-supplemented diet for 45 days, compared to control fish. The results suggest that *U. ohnoi* at low inclusion level could protect the intestinal epithelium of fish, but a relatively short administration period is recommended with the aim of avoiding negative effects on Senegalese sole growth and proteolytic digestive enzyme activities.

1. Introduction

Macroalgae have been widely evaluated in the recent years as a novel resource for aquafeeds [1–4]. Although the protein content of wild-harvested seaweeds is low compared to regular ingredients, macroalgae are rich in biologically active compounds, such as polysaccharides, pigments, polyphenols, and vitamins, which might exert certain beneficial effects on farmed fish [3,4]. Since Mustafa and Nakagawa [5] summarized the role of macroalgae in fish nutrition, numerous studies have evaluated their potential not only as dietary protein source, but also as functional ingredients in practical diets for a variety of fish species [6–8]. Regarding *Ulva* species, abundant literature assessing their effects as diet ingredient on a variety of fish species (Nile tilapia [9,10], gilthead seabream [1,11,12], European seabass [13], rainbow trout [14], and Senegalese sole [3]) is available. Overall, those studies have focused on growth performance, survival rate, nutrient utilization, intermediary metabolism, oxidative status, gut microbiota, intestinal histomorphology, hypoxia tolerance, and flesh quality traits. Most of those investigations evidenced promising results attributable to the inclusion of *Ulva* biomass in aquafeeds, but in some cases low growth performance was also reported when dietary level exceeded 15% [15,16]. Some authors have suggested the presence of antinutritional compounds in *Ulva* as the factors responsible for such growth decrease [9,17–20].

Plenty of antinutritional factors (such as lectins, protease inhibitors, goitrogens, allergens, anti-vitamins, saponins, tannins, phytates, etc.) are present in a vast range of plants and algae [9]. These substances might interfere with the digestibility and bioavailability of nutrients

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[3,17], and also cause morphological changes in the gut. Sáez et al. [18] reported that *Ulva rigida* contains anti-nutrients capable of decreasing alkaline proteolytic activity in juvenile gilthead seabream. Although generalised inhibition of intestinal proteases was reported in that study, apparently fish were able to overcome such interference, as suggested by a lack of effect on growth parameters. In this regard, the assessment of digestive enzyme activities, the analysis of ultra-structural changes of the intestinal mucosa, as well as the relative expression of genes involved in the functionality and integrity of the intestinal epithelium are valuable means for assessing the fish nutritional status and the ability of animals to cope with dietary changes [20–24].

Senegalese sole (*Solea senegalensis*) is a valuable fish species when it comes to diversifying of the European marine aquaculture, owing to high market acceptability and commercial value compared to other marine fish species. Moreover, it has been described that this flatfish species can grow on diets made up of high levels of plant protein [25]. However, the effect of the dietary inclusion of seaweeds for feeding Senegalese sole juveniles has been scarcely evaluated. To the best of our knowledge, with the exception of the study by Moutinho et al. [3], the effects of *Ulva* dietary supplementation have not been assessed before. These authors conducted a long-term feeding trial (up to 9 months), reporting no detrimental effects on growth performance, nutrient utilization, or intestinal morphology attributable to 10% dietary inclusion of *U. rigida*. Nonetheless, further studies are required with lower inclusion levels (< 10%) in order to ascertain aspects that remain to be clarified. The level was set at 5% dietary inclusion in order to elucidate the potential of *U. ohnoi* as functional ingredient for *Solea senegalensis*. Previous studies conducted on *Ulva* species as supplement in fish feeds reported that the best performance was obtained for 5% algae inclusion and decreased at 10 to 20% levels [15,26,27].

This work was aimed at assessing the effects of 5% *Ulva ohnoi* included in diets for Senegalese sole juvenile on growth, digestive enzyme activities, relative expression of genes involved in the functionality and integrity of the intestinal epithelium, as well as on the ultrastructure of the intestinal mucosa of fish.

2. Materials and methods

2.1. Macroalgae and experimental diets

Ulva ohnoi (Hiraoka and Shimada strain UOHN120810) was isolated as a single thallus from the outlet channel of the IFAPA El Toruño facilities (37°12′ 57.39″ N, 7°5′ 5.29″ E), and maintained in culture. Vegetative clones of this fragment were maintained in culture in filtered seawater enriched with f/2 medium. For molecular identification, sequences of the chloroplast-encoded RuBisCo gene (rbcL) and ITS2 were compared with GenBankTM sequences archived by NCBI (https://ncbi.nlm.nih.gov/genbank/). GenBank's BLAST search identified all samples as 100% agreement with *Ulva ohnoi* sequences in the database (GenBank submission pending, Malta et al. in prep.). The biomass needed to prepare *Ulva* diet (U-5) was obtained from up-scaled stock cultures, carried out in 1000 L tanks. *Ulva* biomass (1 kg m^{-3}) was grown in filtered (0.2 μm) natural seawater enriched with f/2 medium [28] with 1.8 mM nitrate and 0.1 mM phosphate, under natural photoperiod. After two weeks, algae were harvested, rinsed with tap water, freeze-dried and kept in a dry place until being used as ingredient in the experimental diets.

Two isonitrogenous (55% on dry weight basis) and isolipidic (15% on dry weight) experimental diets were elaborated by LifeBioencapsulation SL (Spin-off, Universidad de Almería, Spain). The diet designed as U-5 included 5% (w/w) dry *U. ohnoi* biomass. An algae-free diet was used as control (CT). The ingredient composition of the experimental diets is shown in Table 1. Feed ingredients were finely ground and mixed in a vertical helix ribbon mixer (Sammic BM-10, 10-L capacity, Sammic, Azpeitia, Spain) before adding fish oil and diluted choline chloride. All the ingredients were mixed together for 15 min, and then water (300 mL kg^{-1}) was added to the mixture to obtain homogeneous dough. The dough was passed through a single screw laboratory extruder (Miltenz 51SP, JSConwell Ltd., New Zealand), to form 1.1 and 1.9 mm pellets. The extruder barrel consisted of four sections and the temperature profile in each section (from inlet to outlet) was 100 °C, 95 °C, 90 °C and 85 °C, respectively. Finally, pellets were dried in a 12 m^3-drying chamber with forced-air circulation (Airfrío, Almería, Spain) at 30 °C for 24 h, and then kept in sealed plastic bags at −20 °C until use.

Table 1
Ingredient composition of the experimental diets used in the feeding trial.

Ingredients (g kg^{-1} DM)	CT	U-5
Fishmeal LT[b]	674	660
Ulva ohnoi meal[a]	–	50
Squid meal[c]	50	50
Fish protein hydrolysate[d]	50	50
Krill meal[c]	10	10
Shrimp meal[c]	10	10
Gluten meal[c]	20	20
Soybean protein concentrate[e]	20	20
Fish oil	30	29
Soybean lecithin	20	20
Maltodextrin	46	11
Choline chloride[f]	10	10
Vitamin and mineral premix[g]	30	30
Guar gum[c]	15	15
Alginate[c]	15	15

[a] *Ulva* meal (% dry matter): 19.2 ± 1.5 crude protein; 3.2 ± 0.3 crude lipid; 29.9 ± 1.8 carbohydrate; 28.1 ± 1.2. CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively.
[b] (69.4% crude protein, 12.3% crude lipid), Norsildemel (Bergen, Norway).
[c] LifeBioencapsulation SL (Almería, Spain).
[d] (81% crude protein, 8.8% crude lipid) Sopropeche (France).
[e] (65% crude protein, 8% crude lipid) DSM (France).
[f] Sigma-Aldrich (Madrid, Spain).
[g] Mineral and vitamin premix according to Vizcaíno et al. [24]. Proximate composition of control diet: 55.2% crude protein, 12.4% crude lipid, 3.0% fibre, 12.8% ash), and *Ulva* diet: 55.0% crude protein, 12.0% crude lipid, 3.3% fibre, 13.1% ash.

2.2. Fish maintenance and sampling protocol

One thousand five hundred juvenile Senegalese sole (*Solea senegalensis*) (10.7 ± 2.9 g, average initial body weight) were obtained from a commercial hatchery (Cupimar S.A., San Fernando, Cádiz, Spain) and transported to the research facilities of IFAPA El Toruño (El Puerto de Santa María, Cádiz, Spain). Fish were stocked at 1.5 kg m^{-2} in 6 tanks (400 L and 1.58 m^{-2}) connected to a closed recirculation system consisting of a mechanical filter, a skimmer, ultraviolet lights and a biofilter. Fish were fed daily at 2% fish biomass with an experimental diet considered as control diet for 10 days for acclimatizing the fish to the experimental conditions. After the acclimation period, the dietary treatments were randomly assigned to triplicate groups. Thereafter, fish were fed with two different experimental diets (Table 1): control diet (CT diet) and diet containing *U. ohnoi* 5% (U-5 diet) for 90 days at a rate of 3% of their body weight. For estimating the feed intake, the uneaten feed was carefully collected by siphoning 60 min after the administration, then dried for 12 h at 110 C, and weighed. Different parameters were monitored throughout the trial, namely temperature (19.9 ± 0.7 °C), pH (7.8 ± 0.2), salinity (25.7 ± 1.5‰), oxygen concentration (7.0 ± 0.4 mg L^{-1}), nitrite and ammonia (both below 0.1 mg L^{-1}).

Halfway (45 d) through the feeding trial, fish were starved for 12 h and withdrawn in order to carry out the initial measurement of digestive enzyme activities. Animals were taken from their respective tanks (six fish per tank), and then euthanized by clove oil overdose (200 ppm)

followed by spine severing. Whole intestines were randomly grouped (2 intestines were pooled to prepare each enzyme extract). At the end of the feeding trial, fish were fasted for 12 h before sampling. Animals were taken from their respective tanks (six fish per tank), and then euthanized as detailed before. Specimens were then individually weighed and eviscerated. In this case, each intestinal sample was differentiated in anterior and posterior intestine, and then intestinal tissue and luminal content of intestine were processed separately. Intestinal samples were homogenized with ice-cold distilled water (w/v 1: 2). Supernatants were obtained after centrifugation (13,000*g*, 12 min, 4 °C) and stored at −20 °C for further enzymatic analysis.

For gene expression analysis, six animals (2 fish per tank × 3 tanks) per diet were used. Intestine and liver samples were aseptically removed, and the intestine was divided in two equal length sections (named as anterior and posterior) and stored separately. Samples were immersed in TRIsure (Bioline, Meridian Bioscience, London, UK) and immediately stored at −80 °C until further analysis. Finally, the intestines of three specimens from each tank were collected for further examination under light (LM), transmission (TEM) and scanning (SEM) electron microscopy.

2.3. Growth performance calculations

Growth and nutrient utilization were estimated using several morphometric and biometric indices. Final body weight (FBW, g); weight gain ratio (WGR, %) = [(Wf – Wi) / Wi] ∗ 100, where Wf and Wi were final and initial weight (g); specific growth rate (SGR, %) = [Ln (Wf) – Ln (Wi) / days] × 100; feed conversion ratio (FCR) = total feed intake in dry basis (g) / wet weight gain (g).

2.4. Analysis of digestive enzyme activities

Total alkaline protease activity in digestive extracts was measured spectrophotometrically following the procedure described by Alarcón et al. [20], using 5 g L^{-1} casein in 50 mM Tris-HCl (pH 9.0) as substrate. One unit of total protease activity was defined as the amount of enzyme that released 1 μg of tyrosine per min in the reaction mixture, considering an extinction coefficient of 0.008 $\mu g^{-1} mL^{-1} cm^{-1}$ for tyrosine, measured at 280 nm wavelength. Trypsin and chymotrypsin activities were assayed using 0.5 mM BAPNA (N-α-benzoyl-DL-arginine-4-nitroanilide) as substrate according to Erlanger et al. [29], and 0.2 mM SAPNA (N-succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide) according to Del Mar et al. [30], respectively, in 50 mM Tris-HCl buffer, pH 8.5, containing 10 mM $CaCl_2$. Leucine aminopeptidase activity was determined by using 2 mM L-leucine-*p*-nitroanilide in 100 mM Tris-HCl buffer, pH 8.8, as substrate [31], and alkaline phosphatase was assayed using *p*-nitrophenyl phosphate in 1 M diethanolamine, 1 mM $MgCl_2$ buffer, pH 9.5 [32] as substrate. For trypsin, chymotrypsin, and leucine aminopeptidase activities, one enzyme activity unit (U) was defined as the amount of enzyme that released 1 μmol of *p*-nitroanilide per min, considering an extinction coefficient of 8800 M cm^{-1}, measured at 405 nm. For alkaline phosphatase, one activity unit was defined as the amount of enzyme that released 1 μg of nitrophenyl per min considering an extinction coefficient of 17,800 M cm^{-1} for *p*-nitrophenol, measured at 405 nm. All assays were performed in triplicate, and specific enzymatic activity was expressed as units (U) g $tissue^{-1}$. In addition, digestive proteases were separated and visualized in substrate-SDS-PAGE electrophoresis gels following the procedure describe by Laemmli [33]. Zymograms revealing protease active fractions were carried out according to Alarcón et al. [20].

2.5. Assessing the presence of protease inhibitors in diets

The potential inhibitory effects of crude *U. ohnoi* biomass and the experimental diets on Senegalese sole digestive alkaline proteases were evaluated as described in Alarcón et al. [34]. Inhibitory extracts were prepared from both seaweed meal and finely ground diets by homogenizing samples in five volumes of distilled water, and shaking them for 120 min at room temperature, and then for 22 h at 4 °C. Afterwards, the mixtures were centrifuged (20 min, 13,000*g*, 4 °C) and supernatants were stored at 4 °C until use.

The inhibitory effect of the extracts thus prepared on intestinal protease activity was assessed by measuring the remaining protease activity in digestive enzyme extracts after pre-incubation with different volumes of inhibitory extracts (4 °C, 60 min). Controls were prepared by adding distilled water instead of inhibitory extracts. Enzyme inhibition was expressed as percentage of protease inhibition after comparing with a control assay carried out without any inhibitory extract.

2.6. Gene expression analysis

Total RNA was extracted with TRIsure, according to the manufacturer instructions. RNA quality was checked by running an aliquot on agarose gel electrophoresis, and quantified spectrophotometrically (Nanodrop One, Thermo Fisher Scientific, Madrid, Spain) via A260/280 nm and A260/230 nm readings. DNase treatment (Thermo Scientific, Madrid, Spain) was carried out to ensure DNA removal. One microgram of total RNA per sample was reverse transcribed into cDNA using a commercial kit (First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Madrid, Spain). One microliter of each cDNA synthesis reaction was used as template in qPCR reactions carried out to analyse each gene transcription. Six sample replicates were measured. Primers sets used to detect expression of reference genes (*actb2* and *rps4*), intestinal epithelium functionality (*ampn*, *ialp*), lipid absorption and metabolism (*fas*, *cpt1*, *apoa4*, *mtp*, *cd36*, *fabp1* and *fabp2a*), protein metabolism (*tryp1c* and *pept1*), heat shock proteins and oxidative stress (*hsp70*, *hsp90aa*, *hsp90ab* and *sodmn*) are listed in Table 2. Reference gene stability was verified in treated and non-treated samples by means of RefFinder software, a platform integrating Delta CT [35], BestKeeper [36], Normfinder [37] and Genorm [38] computational algorithms, and the overall stability of candidate genes was also determined.

Amplifications were performed in a final volume of 10 μL in triplicate 96-well plates. Reaction mixtures consisted of 5 μL of SsoAdvanced SYBR Green Super-mix (Bio-Rad Laboratories, Hercules, CA, USA), 1 μL of each primer set (10 mM), 1 μL template cDNA and 3 μL DEPC-water. Real time PCR determinations were carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). An initial denaturing cycle of 95 °C for 60 s was followed by 40 cycles of 95 °C for 30 s, 68 °C for 40 s and 72 °C for 60 s. Amplification was followed by a standard melting curve from 65 °C to 95 °C, in 0.5 °C increments for 5 s at each step, in order to confirm that only one product was amplified and detected.

The eventual change in gene expression was calculated by using $2^{-\Delta\Delta CT}$ method [39] normalizing with geometric average of two reference genes (*actb2* and *rps4*) and relative to seaweed-free diet fish from control group.

2.7. Intestine and liver histology

With the aim of assessing potential effects of the experimental diets on the morphology of the anterior intestine and liver of fish, samples were fixed for 24 h in phosphate-buffered formalin (4% v/v, pH 7.2), dehydrated and embedded in paraffin according to standard histological techniques, as described in Vizcaíno et al. [24]. Samples were cut in 5 μm transversal sections and the slides were stained with haematoxylin-eosin (H&E). The stained preparations were examined under light microscope (Olympus ix51, Olympus, Barcelona, Spain) equipped with a digital camera (CC12, Olympus Soft Imaging Solutions GmbH, Muenster, Germany). Images were analysed with specific software (Image J, National Institutes of Health, USA). The length of mucosal folds and total enterocyte height (20 independent measurements per treatment) were determined in intestinal samples.

Table 2
List of *Solea senegalensis* gene primers used in this study.

Code	Gene	Sequence (5′➔3′)	Amplicon size (bp)	Reference
actb2	Beta actin 2	AATCGTGACCTCTGCTTCCCCCTGT TCTGGCACCCCATGTTACCCCATC	113	Infante et al. [40]
rps4	Ribosomal protein S4	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83	Infante et al. [40]
apoa4	Apolipoprotein A-IV	AGGAACTCCAGCAGAACCTG CTGGGTCATCTTGGAGAAGG	122	Bonacic et al. [41]
mtp	Microsomal triglyceride transfer protein	CAGGCGTACACCACATGTAAA GTGATCAGGCTTCTGCAGTG	150	Bonacic et al. [41]
cd36	Cluster of differentiation 36 family	TGAATGAGACGGCTGAGTTG TGTTGTTTCTGCTCCTCACG	168	Bonacic et al. [41]
fabp1	Fatty acid binding protein 1	GCTCATCCAGAAAGGCAAAG GGAGACCTTCAGCTTGTTGC	199	Bonacic et al. [41]
fabp2a	Fatty acid binding protein 2a	ACACACATGACCTTAGCACACTG TGCGAGTATCAAAATCCGGTA	70	Bonacic et al. [41]
fas	Fatty acid synthase	CACAAGAACATCAGCCGAGA GAAACATTGCCGTCACACAC	197	Bonacic et al. [41]
cpt1	Carnitine palmitoyl transferase 1	TAACAGCCACCGTCGACATA AGCGATTCCCTTGTGTCACT	156	Bonacic et al. [41]
ampn	Aminopeptidase N	CTGGCGTGGGACTTTGTGCGAGAT CCGTTGATGAGGTTGGAGAAGGAGAAGG	89	Canada et al. [42]
ialp	Intestinal alkaline phosphatase	GTTGACCAGCAGATGCCAGACAG CAGAACAGATTTGACCTCATTCCCGATA	147	Canada et al [42]
tryp1c	Trypsinogen1C	TCTGCGCTGGATACCTGGAGGGA GCAGCTCACCGTTGCACACAACA	81	Canada et al. [42]
pept1	Enterocyte membrane peptide transporter 1	TCAGGACCATCAGGAGAAGCAGAGG AACACAATCAGAGCTACCACCATGAGAG	195	Canada et al. [42]
hsp70	Heat shock protein 70	GCTATACCAGGGAGGGATGGAAGGAGGG CGACCTCCTCAATATTTGGGCCAGCA	119	Salas-Leiton et al. [43]
hsp90aa	Heat shock protein 90AA	GACCAAGCCTATCTGGACCCGCAAC TTGACAGCCAGGTGGTCCTCCCAGT	105	Manchado et al. [44]
hsp90ab	Heat shock protein 90AB	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA GCCAAGGGGCTCACCTGTGTCG	148	Manchado et al. [44]
sodmn	Superoxide dismutase Mn	GAAAATGTCTGCTGCCACTGTAG GTCTTCCACTCTCCTTCTCAAAGC	77	Jiménez-Fernández et al. [45]

2.8. Ultrastructure of the intestinal mucosa

For TEM observations, samples were fixed in 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5, for 4 h at 4 °C. Then, samples were washed with PBS three times for 20 min each. A post-fixation step with 20 g L^{-1} osmium tetroxide for 2 h was carried out, and tissues were dehydrated by consecutive immersions (20 min each) in gradient ethanol solutions (from 50% to 100%, v/v). Samples were embedded in a mixture (1:1) of absolute ethanol and EPON resin for 2 h under continuous shaking and then included in pure EPON resin and let polymerize for 24 h at 60 °C. The ultra-fine cuts were placed on a 700 Å copper mesh and stained with uranyl acetate and lead citrate. Specimens were observed with a Zeiss 10C (Carl Zeiss, Barcelona, Spain) transmission electron microscope set at 100 KV and 16,000 magnifications.

Samples for SEM were previously washed with 1% *S*-carboxymethyl-L-cysteine (Sigma) for 20 s to remove epithelial mucus, and then fixed for 24 h in phosphate-buffered formalin (4% v/v, pH 7.2). After washing and progressive dehydration in graded ethanol, samples were critical point dried (CDP 030, Leica Microsystems, Madrid, Spain) with absolute ethanol as the intermediate fluid and CO_2 as the transition fluid. Dried samples were mounted on stubs, fixed with graphite (PELCO® Colloidal Graphite, Ted Pella Inc., Ca, USA), and gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). Finally, all samples were screened with a scanning electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). All TEM and SEM digital images were analysed with specific software (ImageJ version 1.45). Microvilli length, microvilli diameter and the number of microvilli over 1 µm distance [46] where determined on TEM images, whereas enterocyte apical area [47] was estimated on SEM images.

2.9. Statistical analysis

Results obtained by real-time PCR were analysed with specific software (XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel, Addinsoft, Paris, France). All data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Differences between the two experimental diets were determined by Student's *t*-test comparison of means. Significance was set for $p < 0.05$.

3. Results

3.1. Growth performance and nutrient utilization

Fish mortality was below 5% in all tanks. Fish body weight increased at least 4 fold their initial values (4.5 and 4.1 folds for CT and U-5, respectively) at the end of the feeding trial (90 d). Fish fed with U-5 diet showed significantly ($p < 0.05$) lower growth parameters after 45 and 90 d compared to control fish (Table 3). Fish accepted the experimental diets, and feed intake was not significantly affected by the inclusion of *Ulva* meal. No significant differences in FCR were observed between treatments at day 90.

3.2. Digestive enzyme activities

Enzyme activities measured in the intestinal extracts of Senegalese sole specimens fed with the experimental diets for 45 and 90 d are shown in Tables 4 and 5, respectively. The inclusion of *U. ohnoi* decreased significantly trypsin, chymotrypsin and total alkaline protease activities in fish fed with U-5 diet for 45 d, whilst these animals showed increased alkaline phosphatase activity. Significantly lower ($p < 0.05$) trypsin and chymotrypsin activities were found in fish fed with U-5 diet for 90 d compared to control group, especially in the anterior intestine.

Table 3
Effects of dietary inclusion of *U. ohnoi* on growth performance of *S. senegalensis*.

	45 days		90 days	
	CT	U-5	CT	U-5
FBW (g)	28.2 ± 0.8 b	24.9 ± 0.6 a	55.5 ± 1.2 b	50.1 ± 1.1 a
WGR (%)	129.6 ± 0.8 b	101.7 ± 5.1 a	352.0 ± 9.5 b	307.9 ± 8.9 a
SGR (%)	1.9 ± 0.1 b	1.6 ± 0.1 a	1.7 ± 0.1 b	1.5 ± 0.1 a
FCR	0.9 ± 0.1 b	1.1 ± 0.1 a	1.0 ± 0.1	1.1 ± 0.1

CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively. Values are mean ± SE of triplicate tanks. Values in the same row with different lowercase indicate significant differences at each sampling time ($p < 0.05$).

Table 4
Enzyme activities (U g tissue^{-1}) measured in the whole intestinal extracts of Senegalese sole juveniles fed with the experimental diets during 45 days.

	CT	U-5
Total alkaline protease	688.6 ± 52.0 b	560.9 ± 23.4 a
Trypsin (×10^{-2})	15.8 ± 1.0 b	5.9 ± 0.1 a
Chymotrypsin	3.3 ± 0.1 b	2.8 ± 0.1 a
Leucine aminopeptidase (×10^{-1})	2.1 ± 0.2	2.7 ± 0.3
Alkaline phosphatase	8.8 ± 0.5 a	14.2 ± 0.9 b

CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively. Values are mean ± SE of triplicate determinations. Values in the same row with different lowercase indicate significant differences ($p < 0.05$).

Total alkaline protease activity was also lower in U-5 group, although significant differences were observed only in the posterior intestine. Alkaline phosphatase activity was significantly higher ($p < 0.05$) in fish fed with U-5 diet compared to CT group at either sampling time (45 and 90 d).

Zymograms of the intestinal proteases of Senegalese sole fed with the experimental diets revealed the same profile of active fractions in both CT and U-5 groups (Fig. 1). However, *U. ohnoi*-enriched diet reduced the intensity of some proteolytic fractions in the posterior intestine sampled at day 90.

The inhibition assay confirmed the presence of protease inhibitors in crude *U. ohnoi* biomass able to inhibit up to 70% of *S. senegalensis* intestinal alkaline proteolytic activity (Fig. 2). Protease inhibition due to the diet containing *U. ohnoi* was significantly lower compared to crude *U. ohnoi* biomass, given that inhibition reached only 12%.

3.3. Transcription of genes involved in protein and lipid metabolism

No decreased transcription of the genes coding for intestinal alkaline phosphatase (*ialp*) and aminopeptidase N proteins (*ampn*) was observed in specimens fed on the diet containing *U. ohnoi* (Table 6). On the contrary, *ampn* up-regulation was detected in the anterior and posterior intestinal sections after feeding for 45 and 90 d, respectively. Regarding *ialp*, significantly increased transcription was detected in the anterior intestine after feeding 90 d with U-5 diet for 90 d.

Transcription of *cd36*, *fabp1* and *mtp* genes, involved in lipid absorption and metabolism, was changed in both anterior and posterior intestinal sections (Table 6), whereas genes related to protein metabolism were modified only in posterior sections after being fed with U-5 diet for 90 d. On the other hand, no changes in gene transcription were recorded for *fabp2*, *apoa4*, *fas* and *cpt* genes in *S. senegalensis* specimens fed with the U-5 diet.

Finally, no changes were detected with regard to the transcription of genes related to stress in hepatic tissues, although *fas* transcription was down-regulated in fish receiving U-5 diet for 45 d (Table 7).

3.4. Histological analysis of intestinal and hepatic tissues

The histological characteristics of liver sections from fish receiving the two dietary treatments are shown in Fig. 3. No severe evidence of necrosis or steatosis was found. However, moderate hepatocyte vacuolization was observed in several samples from specimens fed with CT and U-5 diets for 45 d.

Histological modifications suggesting intestinal damage attributable to the dietary treatments were not found in intestine preparations at any sampling time (Fig. 4). Neither lipid droplet accumulation in the intestinal epithelium nor inflammatory changes in the *lamina propria* were observed. Morphometric analysis revealed a significant increase in fold length and enterocyte height in fish fed with U-5 diet for 45 d (Table 8); however, fish sampled after 90 d showed similar values for both parameters in CT and U-5 groups.

3.5. Ultrastructural study

TEM and SEM observations suggest a lack of alterations in the intestinal brush border (Fig. 5) attributable to the experimental diets. A well-defined and organised brush border membrane was observed in fish fed with U-5 diet. The morphometric analysis of TEM and SEM images revealed that feeding with U-5 diet for 45 d yielded significant increases in both the enterocyte apical area (EA) and the enterocyte absorption surface (TAS) (Table 9). However, similar values were observed after 90 d for both dietary treatments.

4. Discussion

The dietary inclusion of *Ulva ohnoi* led to differences in growth performance and digestive functionality of *Solea senegalensis* juveniles compared to fish fed on control diet. Several studies have reported that the incorporation of *Ulva* sp. at high level in diets can affect negatively fish performance [6,15]. In the present study, 5% *U. ohnoi* diet reduced fish performance after a 3-month feeding trial. However, Moutinho et al. [3] reported that the dietary inclusion of 10% *U. lactuca* over 5 months (from 23 g up to 60 g body weight) did not cause any detectable impact on growth performance and feed utilization in juvenile Senegalese sole.

In the present study the temperature set during the extrusion process of the experimental feeds does not seem to be capable to completely inactivate the macroalgae protease inhibitors. Stronger thermal

Table 5
Enzyme activities (U g tissue^{-1}) measured in fractionated intestinal extracts of Senegalese sole juveniles fed the experimental diets during 90 days.

	Total alkaline protease				Trypsin (×10^{-2})				Chymotrypsin (×10^{-1})				Leucine aminopeptidase (×10^{-1})				Alkaline phosphatase			
Anterior intestine																				
Intestinal tissue																				
CT	151.7	±	17.2		6.1	±	0.2	b	8.0	±	0.3	b	0.9	±	0.1	a	2.1	±	0.1	a
U-5	146.5	±	6.26		4.3	±	0.4	a	2.6	±	0.3	a	1.6	±	0.1	b	2.5	±	0.1	b
Gut content																				
CT	526.8	±	4.7		12.2	±	0.5	b	13.1	±	0.8	b	1.5	±	0.1		1.1	±	0.1	
U-5	435.1	±	7.4		7.0	±	1.0	a	8.4	±	0.9	a	1.3	±	0.3		1.2	±	0.3	
Posterior intestine																				
Intestinal tissue																				
CT	229.8	±	61.9	b	5.3	±	1.1		12.9	±	0.5		2.1	±	0.1		1.7	±	0.1	a
U-5	63.5	±	42.0	a	6.4	±	1.0		10.7	±	0.6		2.5	±	0.1		2.1	±	0.2	b
Gut content																				
CT	498.2	±	50.2	b	7.9	±	0.1	b	11.4	±	0.2		2.5	±	0.4		3.0	±	0.5	
U-5	282.2	±	32.7	a	5.0	±	0.3	a	11.6	±	0.4		2.3	±	0.3		3.2	±	0.1	

CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively. Values are mean ± SE of triplicate determinations. Values in the same column with different lowercase indicate significant difference ($p < 0.05$).

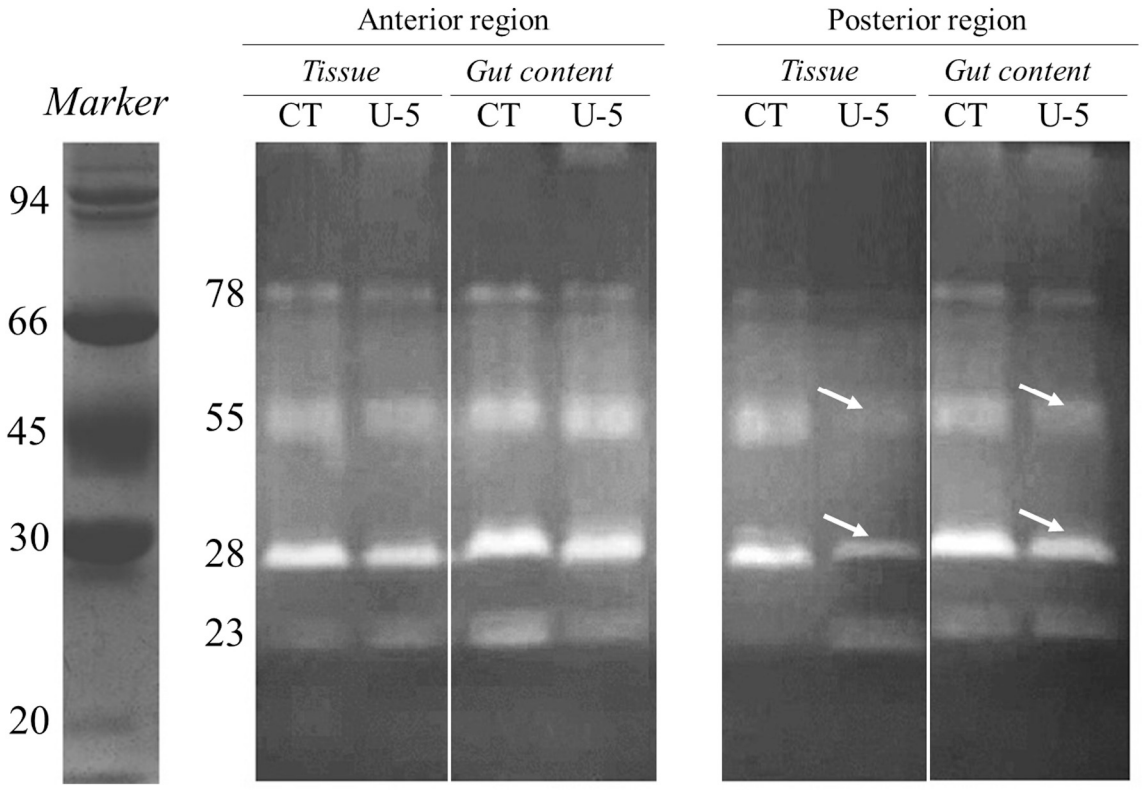

Fig. 1. Zymograms showing intestinal proteases of juvenile *S. senegalensis* fed with the experimental diets at the end of the feeding trial (90 days). CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively. Protein standards (marker lane) were phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), and soybean trypsin inhibitor (20). Five microlitres of the molecular weight marker were loaded. Arrows denote slightly inhibition of two active fractions of the posterior intestine of Senegalese sole.

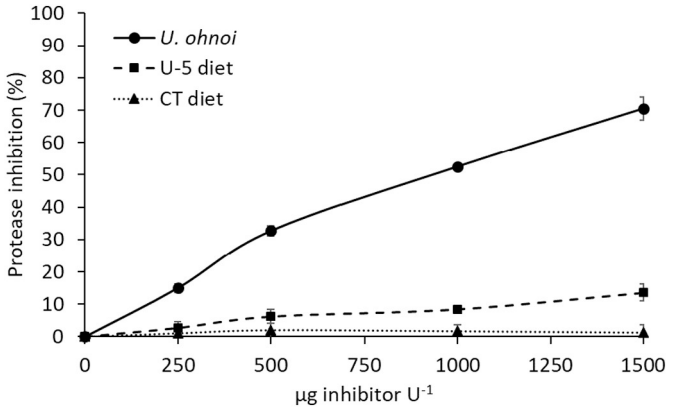

Fig. 2. Dose-response inhibition curve obtained against increased concentrations of crude *U. ohnoi* and experimental diets (µg crude *U. ohnoi* or µg aquafeed per unit (U) of alkaline protease activity). CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively. Each point represents average values of three replicates ± SE.

treatment of raw *U. ohnoi* might well be needed for inactivating such anti-nutritional factors. However, excessive heat treatment could damage essential amino acids and other bioactive molecules, and also reduce nutrient availability. Nevertheless, the disparate response of fish after *Ulva* dietary administration described in the literature might be related to differences in factors such as fish physiological maturity, the specific strain of *Ulva* used, the length of the feeding trial, and even the inclusion level of the seaweed in diets.

Feed efficiency is mainly related to the physiological capacity of fish to digest and transform the ingested nutrients [48], and this ability largely relies on the presence of an adequate set of digestive enzymes.

Table 6
Relative transcription of genes involved in the intestinal epithelium functionality and integrity (*ampn* and *ialp*), lipid (*cd36*, *fabp1*, *fabp2*, *apoa4*, *mtp*, *fas* and *cpt*) and protein (*pept1c* and *tryp1c*) metabolism in the anterior and posterior intestinal regions of *S. senegalensis* specimens fed on a diet containing *U. ohnoi* (5% w/w) for 45 and 90 days.

Gene	45 days		90 days	
	Anterior intestine	Posterior intestine	Anterior intestine	Posterior intestine
ampn	2.0 ± 0.6*	1.2 ± 0.1	1.5 ± 0.4	2.3 ± 0.3*
ialp	1.8 ± 0.6	1.7 ± 0.5	6.3 ± 2.6*	1.2 ± 0.3
cd36	2.2 ± 0.7 *	1.0 ± 0.4	1.3 ± 0.3	2.7 ± 0.5*
fabp1	3.0 ± 0.9	0.4 ± 0.1	6.1 ± 2.0*	3.1 ± 1.9
fabp2	1.2 ± 0.4	1.3 ± 0.4	1.0 ± 0.4	1.0 ± 0.2
apoa4	1.2 ± 0.3	0.8 ± 0.2	2.4 ± 0.8	2.9 ± 0.6
mtp	1.8 ± 0.5	0.1 ± 0.1*	3.5 ± 0.5*	1.8 ± 0.4
fas	0.8 ± 0.4	4.1 ± 2.1	2.0 ± 0.2	2.3 ± 0.7
cpt	1.1 ± 0.2	1.2 ± 0.3	0.6 ± 0.1	1.3 ± 0.1
pept1c	0.6 ± 0.6	0.7 ± 0.2	1.0 ± 0.2	0.1 ± 0.1*
tryp1c	0.7 ± 0.3	0.3 ± 0.1	0.9 ± 0.2	3.5 ± 0.5*

Fold change values (mean ± SE) are relative to those measured in fish fed on control diet without *U. ohnoi*. Asterisks indicate significant differences ($p < 0.0.5$).

Hence, knowledge on digestive enzyme activities is a key tool when it comes to evaluate new ingredients for aquafeeds [49]. It has been reported that the inclusion of algae in diets can induce changes in the compartmental distribution of specific enzyme activities involved in digestive and absorptive processes [24]. The present study also evidenced differences in activity levels between luminal and mucosal compartments (Table 5). Digestion and absorption of nutrients depend on the activity of the digestive enzymes, in particular those located in

Table 7
Relative transcription of genes involved in stress and lipid metabolism in the liver of *S. senegalensis* specimens fed on a diet containing *Ulva ohnoi* (5% w/w) for 45 and 90 days.

Gene	45 days	90 days
hsp70	1.5 ± 0.2	0.4 ± 0.1
hsp90aa	1.7 ± 0.3	1.0 ± 0.1
hsp90ab	0.7 ± 0.3	1.4 ± 0.3
sodmn	0.9 ± 0.5	0.9 ± 0.3
cpt1	1.6 ± 0.3	1.1 ± 0.2
fas	0.3 ± 0.1*	1.3 ± 0.9

Fold change values (mean ± SE) are relative to those measured in fish fed with control diet without *U. ohnoi*. Asterisks indicate significant differences ($p < 0.0.5$).

the brush border section of the intestine (such as leucine aminopeptidase and alkaline phosphatase), which are responsible for the final stages of breaking down and assimilation of feed ingredients [50]. Previous studies carried out by Vizcaíno et al. [11] found that the inclusion up to 25% of *Ulva rigida* and *Gracilaria cornea* in diets did not cause negative effects on brush border digestive enzymes of gilthead seabream. In the present work, the increased transcription of *ampn* gene in specimens fed on the diet containing *Ulva* was not reflected in higher leucine aminopeptidase activity. However, a significant increase of alkaline phosphatase (both enzyme activity and gene transcription), was observed in fish fed with *Ulva*-supplemented diet. Alkaline phosphatase is considered a key enzyme of the intestinal brush border, often used either as an indicator of the intestinal integrity [51], or as a general marker of nutrient absorption [50]. Hence, increased activity of this enzyme could be symptomatic of improved intestinal absorptive capacity [47,52,53].

Cellular uptake of small peptides is mediated by H^+-coupled peptide transporter (PepT1), located at the brush border membrane of intestinal epithelial cells [54]. PepT1 is a low-affinity, high-capacity transporter that mediates transport of di- and tripeptides from the apical membrane of the epithelial cells into the enterocytes [23]. Several authors have reported the modulation of the protein activity and *pept1* gene expression associated to changes in dietary protein sources [22,23]. In the present work, *pept1* was down regulated in fish specimens fed with U-5 diet for 90 days. This finding might be related to lower peptide availability and might have resulted in decreased peptide transport in the posterior intestinal sections of *S. senegalensis*. Bakke et al. [55] reported altered *pept1* expression in response to changes in luminal protein content and suggested that *pept1* may be regulated among the different segments of the intestinal tract.

The gene *tryp1c* codes for one anionic trypsinogen isoform, which is highly expressed in comparison to the other three trypsinogen types identified in *S. senegalensis* [44]. Trypsinogens are synthesized as proenzymes that are activated later by enterokinase, and transformed into its active form in the intestinal lumen. In the present study, increased expression of *tryp1c* was observed in fish receiving U-5 diet for 90 days. However, decreased proteolytic activity was detected in specimens fed with *Ulva* diet for 45 and 90 d, although this effect was more evident after 90 d. Differences between mRNA levels and trypsin activity observed in other species indicate post-transcriptional or translational regulation of the enzymatic activity [56,57]. Similarly, Gamboa-Delgado et al. [58] observed a lack of correlation between trypsin activity and *tryp1c* transcription in *S. senegalensis* larvae fed different diets. According to Sánchez-Paz et al. [59] changes in mRNA concentration might or might not result in physiologically relevant changes in enzyme concentration. Indeed, lagged response between mRNA synthesis and trypsin activity might occur as well [57].

According to Canada et al. [42] increased *tryp1c* and even *ampn* and *ialp* expression might possibly indicate a strategy to optimize the digestion and utilization of dietary protein. In this regard, it could be expected that changes in mRNA levels respond to modifications in nutrient availability, or to the presence of anti-nutritional factors in feeds, hindering adequate protein digestion. Thus, fish can keep an adequate level of active proteases without compromising digestive and absorptive processes [60]. It has been reported that macroalgae species, including *Ulva* sp., contain several anti-nutritional factors, such as lectins and protease inhibitors [9,18,19,61], that might interfere with feed utilization processes, not least digestion [62]. Indeed, results obtained in the protease inhibition assay confirmed the presence of protease inhibitors in *U. ohnoi* biomass and, to a lesser extent in *Ulva*-supplemented diet, enough to clearly inhibit Senegalese sole intestinal proteases (Fig. 1). Therefore, the increase in *tryp1c* expression could be explained by a positive feedback regulation in the mechanism of protease secretion aimed at coping with the reduction in digestive capacity caused by *Ulva* inhibitors. In mammals, the inhibition of intestinal proteases leads to the stimulation of cholecystokinin secretion and to increased secretion of pancreatic enzymes [63]. However, the results obtained in the present study showed that the stimulation of *tryp1c* expression in Senegalese sole was not reflected in increased proteolytic activity, likely due to the presence of protease inhibitors.

S. senegalensis specimens receiving U-5 diet showed increased *cd36* transcription after 45 and 90 d. The protein cluster of differentiation 36 (CD36), also known as fatty acid translocase, is one of the proteins involved in the active fatty acid (FA) uptake when luminal concentrations are low [64,65]. This fatty acid translocase is expressed in the intestinal brush border, and certain role in lipid sensing has also been attributed to it [66]. Bonacic et al. [41] observed that intestinal *cd36* expression was affected by dietary lipid content, showing lower levels in *S. senegalensis* fed on higher lipid diets. On the other hand, decreased lipase has also been observed in seabass fed on diets supplemented with *Ulva* (2.5%) [7]. Given that lipases are responsible for the hydrolysis of lipids into FA, lower lipase activity could have an impact similar to low lipid diet. Thus, higher *cd36* transcription observed in *S. senegalensis* might represent a response to lower lipid availability of *Ulva* supplemented diet caused by the inhibition of lipase activity, same as reported for Chlorophyta algae [67]. Considering the role of CD36 in FA detection, responses observed might also indicate certain role in nutrient

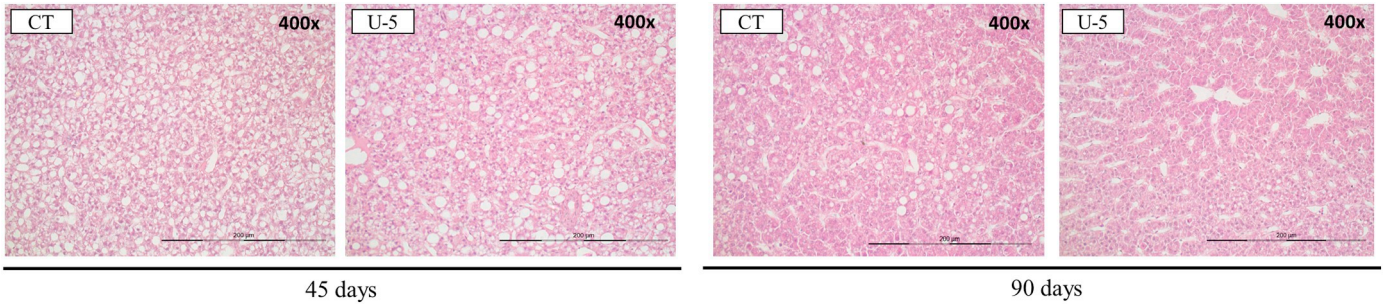

Fig. 3. Light microscopy details of the liver of Senegalese sole juveniles fed on the experimental diets for 45 and 90 days. H&E stain, magnification ×400, scale bar 200 µm. CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively.

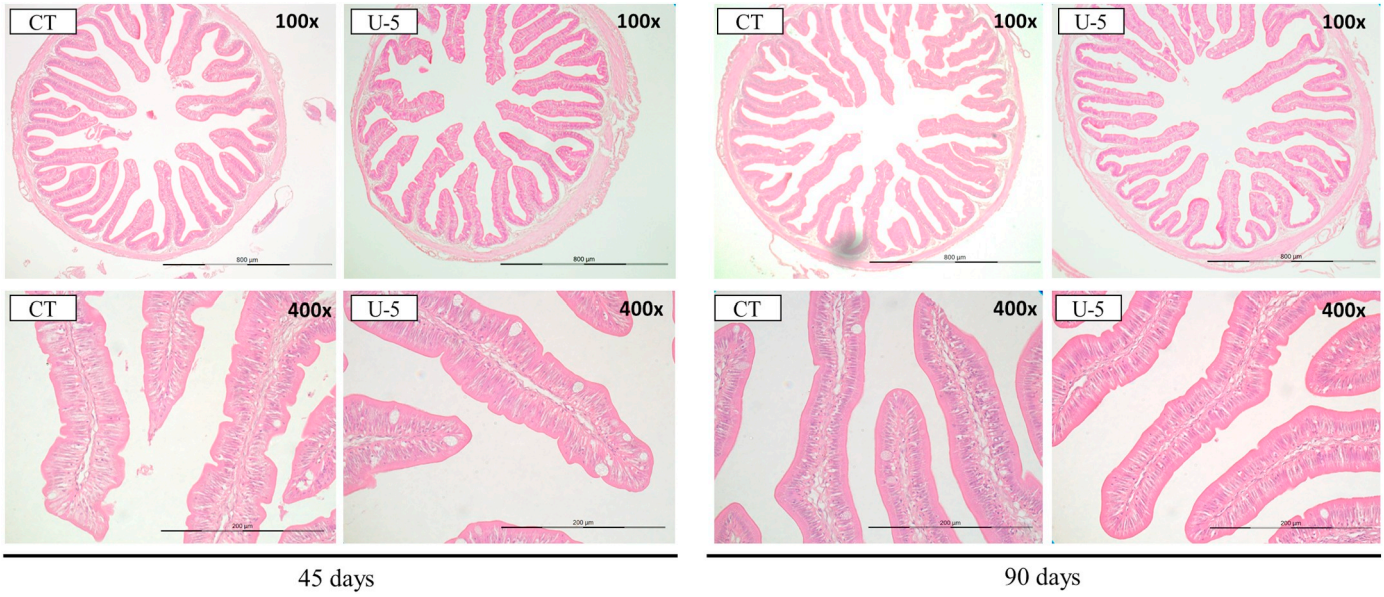

Fig. 4. Light microscopy images from the anterior intestine of Senegalese sole juveniles fed on the experimental diets for 45 and 90 days. H&E stain, magnification ×100, scale bar 800 µm; and ×400, scale bar 200 µm. CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively.

Table 8
Quantification of the histological parameters assessed in the intestine of *S. senegalensis* juveniles fed with the experimental diets during 45 and 90 days.

	Fold length (µm)				Enterocyte height (µm)			
45 days								
CT	421.7	±	16.7	a	31.8	±	0.7	a
U-5	494.1	±	19.0	b	36.1	±	0.7	b
90 days								
CT	395.5	±	13.8		30.7	±	0.7	
U-5	406.2	±	12.5		29.6	±	0.5	

CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively. Values are mean ± SE of triplicate determinations. Values in the same column with different lowercase indicate significant differences ($p < 0.05$).

sensing. However, further studies are necessary to elucidate the meaning of CD36 in *S. senegalensis* gastrointestinal tract.

Mtp is a lumen protein that facilitates lipid binding to lipoproteins by acting as a chaperone, enabling ApoB folding in the endoplasmic reticulum of enterocytes. Contrary to ApoB, Mtp seems to be regulated at transcriptional level [68]. Different responses have been reported depending on the fish species considered. Castro et al. [69] did not find nutritional modulation of Mtp transcript levels in *Dicentrarchus labrax* fed with fish or plant oil. In the present work, contrasting results have been observed in Senegalese sole fed on *Ulva* diet for 45 and for 90 d. Thus, down-regulation was detected in the shorter feeding period, whilst in fish fed on *Ulva* diet for 90 d, increased *mtp* transcription was observed together with up-regulation of *fabp1* and *cd36* genes.

Fabps proteins facilitate the intracellular transport of FA from the microvilli to the endoplasmic reticulum for re-esterification, and they

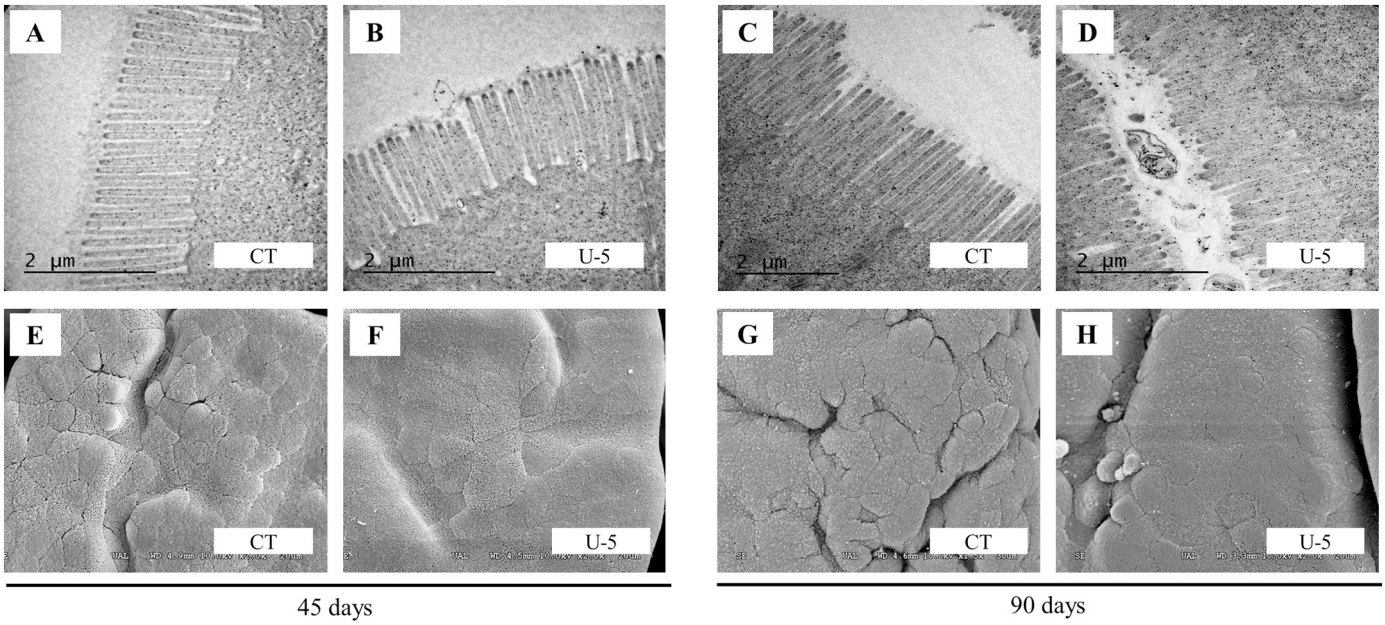

Fig. 5. Comparative TEM and SEM micrographs from the anterior intestinal region of juvenile *S. senegalensis*. (A, B) and (C, D) are TEM images obtained at 45 and 90 days, respectively. (E, F) and (G, H) are SEM images obtained at 45 and 90 days, respectively. CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively.

Table 9
Microvilli morphology of the anterior intestine of Senegalese sole juveniles fed with the experimental diets during 45 and 90 days.

	ML (μm)				MD (μm)				EA (μm^2)				TAS (μm^2)			
45 days																
CT	1.37	±	0.01		0.12	±	0.01		22.6	±	0.4	a	539.2	±	9.7	a
U-5	1.32	±	0.02		0.11	±	0.01		29.1	±	0.9	b	833.7	±	26.8	b
90 days																
CT	1.63	±	0.03	b	0.12	±	0.01	a	17.1	±	0.7		526.3	±	22.4	
U-5	1.21	±	0.02	a	0.14	±	0.01	b	21.7	±	1.6		486.2	±	36.0	

CT and U-5 stand for control and 5% *U. ohnoi* diets, respectively. Values are mean ± SE of triplicate determinations. ML: microvilli length; MD: microvilli diameter; EA: enterocyte apical area; TAS: total enterocyte absorption surface. Values in the same column with different lowercase indicate significant differences at each sampling time ($p < 0.05$).

complement the function of CD36. Increased *fabp* transcription has been correlated with increased amount of lipid droplets in zebra fish enterocytes [70]. On the contrary, neither *fabp1* nor *fabp2* transcription was modified in *S. senegalensis* fed with different lipid levels [41]. In addition, certain role has been proposed for Fabps during lipolysis, enabling rapid transport of FA to the cell membrane to be exported of re-esterified in the endoplasmic reticulum [71]. It could be assumed that the joint up-regulation of *cd36* and *fabp1* may result in increased FA transport from the apical to the basolateral membrane of enterocytes. Then, increased Mtp would facilitate lipid incorporation to lipoproteins. However, considerable variability in individual response was observed in both experimental groups, and it remains to be elucidated whether increased *S. senegalensis fabp1* transcription observed in the present study is related to lipolysis, to increased lipid transport from the intestinal lumen, or associated to a compensatory response to lower dietary lipid availability.

Changes in the expression of genes related to stress have not been observed in the liver of *S. senegalensis* fed with the *U. ohnoi* diet, this fact pointing out to a lack of metabolic alterations in hepatic cells. However, feeding with the diet containing *U. ohnoi* for 45 d led to decreased *fas* transcription. Fatty acid synthase (FAS) is a key enzyme involved in the synthesis of long chain fatty acids [72]. Inhibition of FAS activity and decreased gene transcription have been associated with diets with high fatty acid content in grass carp (*Ctenopharyngodon idella*) and *S. senegalensis* [73,74]. On the other hand, Borges et al. [75] observed enhanced FAS activity in *S. senegalensis* fed with high level of starch in low-fat diets, indicating that digestible carbohydrates promote lipogenesis. In the case of *Ulva*-supplemented diet, lower *fas* transcription could indicate reduced fatty acid synthesis in fish receiving the macroalgae for 45 d. Interestingly, histological examination of the liver revealed moderate presence of lipid vacuoles in both groups of fish after 45 d, whereas no alterations in hepatic lipid metabolism were observed after a longer feeding period (90 d). Despite this, none of the experimental diets used in the present study induced symptoms of steatosis. On the contrary, Gerreiro et al. [1] reported a lack of effect of 5% *Ulva lactuca* on hepatic fatty acid biosynthesis in gilthead seabream juveniles.

Regarding the intestinal morphology, histological observations indicated that the administration of *Ulva*-supplemented diets did not cause enterocyte or gut alterations. The results obtained in this work suggest that the contradictory findings reported in the literature might well be attributed to differences among fish species. Hence, according to previous studies, the effects of macroalgae on gut morphology are species-specific, and they are also related to the dietary inclusion level. Vizcaíno et al. [11] described a lack of intestinal inflammatory changes in juvenile *S. aurata* specimens fed on diets supplemented with *Ulva rigida* and *Gracilaria cornea* biomass up to 25% inclusion level. Silva et al. [9] reported that 10% *Ulva* sp. dietary inclusion did not cause gut morphological alterations in juvenile Nile tilapia, although the same inclusion level of *Gracilaria vermiculophylla* or *Porphyra dioica* led to decreased villi length and intestine diameter in this species.

Electron microscopy analysis of the intestinal brush border did not evidence structural alterations attributable to the dietary inclusion of *U. ohnoi* biomass. Vizcaíno et al. [24] reported positive effects on the absorptive capacity of the anterior intestine in *S. senegalensis* specimens fed with microalgae-supplemented diets. Feeding with *Ulva* diet has been reported to result in modulation of *S. senegalensis* gastrointestinal microbiota. Recently, Tapia-Paniagua et al. [76] observed increased abundance of *Vibrio* spp. in specimens receiving a diet containing *U. ohnoi* (5%) compared to fish fed with the control diet. Increased presence of *Vibrio* in the intestinal microbiota might well be associated to their ability to degrade algal polysaccharides, as suggested by Gobet et al. [77]. *Vibrio* species have diverse enzymes including alginate lyases, chitinases, β-1-3 xylanases, cellulases and also ulvan lyases, among others [78] that enable bacteria to depolymerize a variety of carbohydrates present in *Ulva* cells, which accounts for 30% of dry biomass. We hypothesize that those compounds might promote enhanced growth development of numerous intestinal microorganisms exerting a beneficial effect on the health status of the gut barrier. The intestinal mucosa is the first line of host defence against harmful microbes in the gastrointestinal tract, and the results suggest that the use of *Ulva* might enhance its role in this regard the function of intestinal barrier. In our study, the increased enterocyte absorptive surface observed in Senegalese sole specimens fed with *Ulva*-supplemented diet for 45 d is in agreement with the increased alkaline phosphatase activity measured in the intestinal mucosa of those fish. Moreover, a significant reduction of the pathogen *Tenacibaculum* spp. has been described in the digestive tract of Senegalese sole fed 5% *U. ohnoi*, which seems to reflect that microbiota modulation through dietary additives could limit not only the risk of disease in the fish but also the potential transmission of the pathogen to other hosts [76]. Further studies aimed at fully ascertaining those aspects are still needed.

In short, the inclusion of 5% *U. ohnoi* into juvenile *S. senegalensis* diets led to reduced growth and decreased pancreatic protease activity. Changes observed in the ultrastructural morphology of the intestinal mucosa evidenced an increased absorptive surface after 45 days of administration, but not after 90 days. In addition, *Ulva* supplementation modulated the transcription of genes involved in protein and lipid metabolism. These results suggest that *U. ohnoi* could be used as an additive for improving the intestinal epithelium of Senegalese sole, but a short period of administration is recommended in order to avoid the undesirable effects on growth performance and digestive proteolytic enzyme activities observed when fed to juvenile fish at initial phases of the on-growing period. Further studies are needed to refine both dietary levels and feeding periods of *Ulva*-supplemented feeds, in order to eventually minimize the negative effects observed in *Solea senegalensis*.

Author contributions

A.J. Vizcaíno and M. Fumanal performed the fish sampling. C. Fernández-Díaz and V. Anguis participated in sampling, cultivation of the algae, and fish maintenance. A.J. Vizcaíno, M.I Sáez and T.F. Martínez performed enzymatic and structural analysis. M. Fumanal performed DNA extraction, and data collection. F.J. Alarcón prepared

the aquafeeds. A.J. Vizcaíno, M. Fumanal, F.J. Alarcón, C. Fernández-Díaz and M.C. Balebona performed the data analysis and drafted the manuscript. M.C Balebona, M.A. Moriñigo, and F. J. Alarcón designed the work. All authors critically revised and approved the manuscript.

Statement of informed consent, human/animal rights

The authors state that no conflicts, informed consent, human or animal rights are applicable. All studies involving fish were conducted in accordance with the requirements of the Directive 2010/63/EU, and the Spanish legislation (Real Decreto 53/2013), regarding the ethical rules applicable in research involving laboratory animals. Thereby, all the procedures were authorized by the Bioethics and Animal Welfare Committee of Institute of Agricultural and Fisheries Research and Training (IFAPA).

Declaration of authors' agreement

All authors agree to the authorship and submission of the manuscript to Algal Research for peer review.

Declaration of Competing Interest

The authors declare no conflict of interests.

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