Assessment of dietary inclusion of crude or hydrolysed *Arthrospira platensis* biomass in starter diets for gilthead seabream (*Sparus aurata*).

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Abstract

This work evaluates the effects of the dietary inclusion of crude or hydrolysed *Arthrospira platensis* (Cyanobacteria) biomass on growth, muscle composition, digestive functionality and immune activities in gilthead seabream (*Sparus aurata*) fry (20.32 mg mean body weight). A 40-day feeding trial was conducted, aimed at assessing four experimental diets that included 5 or 10% (w/w) *A. platensis*, either crude or hydrolysed, plus a microalgae-free diet as control batch. Overall, none of the dietary treatments caused negative impacts on fish growth, body composition, muscle fatty acid profile, or innate immune response. Thus, the dietary inclusion of both crude and hydrolysed *A. platensis* reduced significantly the oxidation of muscle lipids, especially when using hydrolysed biomass, regardless of the dietary inclusion level. In relation to digestive enzymes, significantly higher levels of trypsin, chymotrypsin and leucine aminopeptidase activities were measured in fish fed on *A. platensis*-supplemented diets compared to

control fish. In addition, within each inclusion level (5 or 10% w/w), those animals fed with diets that included the hydrolysed biomass yielded consistently higher digestive enzyme activities than those receiving the crude biomass. Microalgae dietary inclusion also induced favourable changes in fish gut morphology, according to the increase in microvilli length and diameter observed. This fact might well have contributed to reinforce the role of the intestinal mucosa as a protective barrier against microorganisms, as well as to enhance the absorptive capacity of the intestinal mucosa. Finally, 10% inclusion of microalgae hydrolysate enhanced lysozyme activity in liver, this fact suggesting improved protection against infectious diseases. In conclusion, the positive effects observed in fish fed with the diets including *A. platensis* up to 10% (not least the hydrolysed biomass) with regard to the different parameters assessed (digestive enzyme activities, intestinal epithelium ultrastructure, muscle lipid oxidation, and lysozyme activity) suggest the benefits of including this product in starter feeds for gilthead seabream fry.

Keywords: *Arthrospira platensis*, microalgae hydrolysate, *Sparus aurata*, starter feed, fish nutrition.

1. Introduction.

Early stages in the life cycle of marine fish are critical for the subsequent developmental changes taking place, in which fish undergo drastic morphological and physiological changes that determine further viability (Torres *et al*., 2020). Besides organ differentiation and morphogenesis, the larval stage is characterized by the highest growth rate throughout the productive cycle of commercial fish (Savoie *et al*., 2011). Consequently, considerable research effort has been made to develop inert microdiets that must fulfill certain premises for adequate larval development. Besides being tasty

and economical, the most important of such requirements is to provide the necessary protein, amino acids, and fatty acids (Conceição *et al*., 2007; Vizcaíno *et al*., 2016; Khoa *et al*., 2019). These requirements have been traditionally met by the use of fishmeal and fish oil as the main protein and fatty acid sources, although plant protein ingredients, such as soybean meal, have also been included in feedstuffs (Ayala *et al*., 2020). However, in recent years, microalgae species and specific strains of cyanobacteria have emerged as a raw material of extraordinary interest in aquaculture (Shah *et al*., 2018; Han *et al*., 2019). Owing to their chemical composition, species of the genus *Arthrospira* are considered excellent candidates that have been successfully used as ingredients in feeds for several fish species (Mahmoud *et al*., 2018; Liu *et al*., 2019; Niccolai *et al*., 2019; Rajasekar *et al*., 2019). Despite this, the relatively high cost of microalgae, compared to conventional ingredients, limits their use in commercial aquafeeds (Yarnold *et al*., 2019). Even though cheaper than other microalgae species commercially available, the current price of freeze-dried *A. platensis* (around 15 USD kg^{-1}) is, by far, above the average price of fishmeal (in the region of 1.6-2.0 USD kg^{-1}), and therefore it is not yet feasible to replace fishmeal as the main ingredient in aquaculture feeds. Hence, the interest of microalgae as potential functional additive in aquafeeds, instead of a major dietary ingredient, is increasing considerably (Galafat *et al*., 2020).

On the other hand, microalgae inclusion in aquafeeds becomes more difficult in the case of early life stages because fish larvae cannot handle dietary components like juveniles. The scarce enzyme activity during these stages hinders digestion processes (Khoa *et al*., 2019). Namely the limited luminal protease activity affects directly protein digestion, a factor that has been pointed out as a major limitation to the utilization of diets by fish larvae (Cahu and Infante, 2001; Cai *et al*., 2015). In this regard, this work raises the question of whether protein hydrolysates obtained from microalgae could be used as dietary ingredient in weaning diets for marine fish larvae. Earlier, Galafat *et al*. (2020) corroborated the potentially positive physiological effects of *Arthrospira* sp. protein

hydrolysates as bioactive additive in diets for juvenile gilthead seabream (*Sparus aurata*). According to this work, an enzymatic pre-treatment could release low molecular weight bioactive peptides and free amino acids that could be easily absorbed by enterocytes, this leading to earlier maturation of digestive organs, as well as to improved nutrient digestibility and acceptability by the animal (Srichanun *et al*., 2014). Beside this, a range of molecules that are known to be bioactive or so-called "nutraceutical" effects. These bioactive compounds might trigger host immune reactions in response to pathogen surface molecules, thus improving anti-inflammatory and antimicrobial abilities of the host (Novak *et al*., 2009), apparently by raising both the non-specific and the specific immunity of fish (Sahoo and Mukherjee, 2001). This fact might well be critical in early life stages in which the specific immunity is not well established, and the immune response of fish depends on non-specific activities (Uribe *et al*., 2011). It has been recently described an immunity communication between gut and liver in which more specific immune proteins and transcript were detected in gut and more non-specific immune molecules in liver (Wu *et al*., 2016). The potential role of microalgae enzymatic hydrolysates in aquafeeds on the abovementioned physiological phenomena at early stages of fish development remains unexplored. Therefore, this research is aimed at assessing the effects of low dietary inclusion levels of crude and hydrolysed *Arthrospira platensis* on fish growth performance, proximate composition, fatty acid composition, muscle lipid oxidation, digestive functionality, and innate immune activities in gilthead seabream fry.

2. Material and methods.

2. 1. Microalgae biomass.

Crude biomass of *Arthrospira platensis* (crude protein: 65% dry weight, DW; crude lipid: 5%, DW) was provided by Biorizon Biotech (Almería, Spain). The term microalgae is

used to refer to prokaryotic species such as cyanobacteria *A. platensis* (Cyanophyceae) with microscopic size. Cyanobacterium *Spirulina platensis* and *A. platensis* are considered as synonyms to each other as indicated by Guiry and Guiry (2018). The microalgal protein hydrolysate was produced from the crude raw biomass after performing an enzymatic hydrolysis following the method described by Saadaoui *et al*. (2019), as modified by Galafat *et al.* (2020). Briefly, a sludge containing 150 g L^{-1} microalgae biomass was incubated with 0.2% w/w mixture of commercial proteases (Alcalase 2.4L® and Flavourzyme 1000L® from Novozymes A/S, Bagsvaerd, Denmark) under controlled conditions (pH 8.0 and 50ºC under continuous stirring) during 4 h. Immediately after the hydrolysis, the reaction mixture was heated at 80°C for 15 min in order to inactivate commercial proteolytic enzymes. The hydrolysate was then kept at 4ºC until use.

2.2. Experimental diets.

Five iso-nitrogenous (59%, DW) and iso-lipidic (18.0%, DW) experimental diets were formulated; two of them contained 5 and 10% w/w microalgae raw biomass (designated as C-5 and C-10, respectively); two other experimental groups included 5 and 10% w/w hydrolysed biomass (designated as H-5 and H-10, respectively), and a fifth diet, microalgae-free, was used as the control batch (CT). The formulation and chemical composition of the experimental diets are shown in Tables 1, 2 and 3. The experimental diets were designed and manufactured by CEIMAR-University of Almería (Service of Experimental Diets) (Almeria, Spain) using standard aquafeed processing procedures. Particle size of the microdiets elaborated ranged from 0.3 to 0.8 mm.

2.3. Fish and experimental design.

Larval rearing took place at the Planta de Cultivos Marinos facility of the Instituto Español de Oceanografía (IEO, Puerto de Mazarrón, Murcia, Spain). Previously, fertilized eggs of gilthead seabream (*Sparus aurata*) were stocked in a 500 L cylindrical-conical

incubator with gentle aeration until hatching. The incubator was supplied with ultraviolet-irradiated seawater at 19±0.5ºC, same as spawning water temperature. Then, hatched larvae were transferred to a 5000-L tank where they began to be fed with rotifers (*Brachionus plicatilis*) once the mouth opening occurred (5 days post-hatching, dph) until 27 dph, at a density of 20 rotifers mL^{-1}. From 20 to 27 dph, Artemia nauplii at a concentration of 1-3 nauplii mL^{-1} were introduced in the tank, and Artemia metanauplii from 26 dph until the weaning period was completed (50 dph). From 40 to 50 dph, larvae were progressively weaned through a co-feeding regime based on Artemia metanauplii and the inert commercial diet (Gemma Wean 0.2, Skretting). The amount of Artemia was progressively reduced from 3 to 0.5 Artemia mL^{-1}, whereas inert feed supply increased. After weaning, gilthead seabream larvae were transferred to 170 L tanks (510 larvae $tank^{-1}$; 3 larvae L^{-1}) in an open circulation system five days before the beginning of the feeding trial. UV sterilized seawater (38‰ salinity; 20-23°C) was provided into the tank system at an exchange rate of 150 L h^{-1}. Supplemental aeration was provided in order to maintain dissolved oxygen above 6.5 mg L^{-1}, and photoperiod was fixed on a 14-h light/10-h dark cycle (450 lx). Ammonia (<0.1 mg L^{-1}), nitrite (<0.2 mg L^{-1}), and nitrate (<50 mg L^{-1}) were determined once weekly.

From 55 dph onwards, larvae (~ 24 mg, average initial body weight) were exclusively fed with the experimental diets to apparent visual satiation (*ad libitum*) six times daily. The different dietary treatments (CT, C-5, C-10, H-5 and H-10) were randomly assigned to the experimental tanks. Each dietary treatment was tested in triplicate (5 feeding treatments × 3 tanks per treatment) for 40 days. Feeding rate and feed size were equal in all tanks, and those were adjusted according to fish age, larval weight, and water temperature, following the recommendations for gilthead seabream fry provided by Skretting España (Burgos, Spain).

2.4. Fish sampling.

At the beginning, larvae (55 dph) were individually weighed and measured after 12-h fasting. During the feeding trial, fasted fish from each tank were randomly sampled at 75 and 95 dph. In each sampling point, 100 fish per tank were weighed, measured and sacrificed by anaesthetic overdose (50 ppm clove oil) according to the requirements of the Council Directive 2010/63/UE. One pool of complete intestines (15 animals) from fish previously fasted for 6-h was prepared from each tank. The biological material was then processed to obtain crude extracts prior to determine digestive enzyme activities. In brief, intestines were manually homogenized in distilled water at 4°C to a final concentration of 0.5 g mL^{-1}. Supernatants were obtained after centrifugation (16,000 ×*g* for 12 min at 4°C) and stored at −20°C until further analysis. For proximate composition analysis, one hundred of 95 dph-fry carcasses (without viscera) per tank were freeze-dried. In addition, muscle samples (1 g) of a pool of ten fish per tank were used for lipid oxidation determinations, and the excess sample was freeze-dried for fatty acid composition. The intestines of three specimens from each tank were collected at 95 dph for examination by transmission (TEM) and scanning (SEM) electron microscopy. Finally, also at 95 dph, the liver of three fish from each tank were extracted, weighed, and individually kept at −80ºC until processing for the analysis of immune-related parameters.

2.5. Growth performance, nutrient utilization and somatic indices.

Growth performance was assessed by different parameters according to the following formulae: daily gain (DG, g day^{-1}) = ($W_f - W_i$) / days; specific growth rate (SGR, %/d) = (Ln (W_f) − Ln (W_i) / days) × 100; condition factor (K) = (W_f / SL^3) × 100, where W_f was the final weight (g), W_i was the initial weight (g) and SL was the standard length. Somatic indices were also calculated: Hepatosomatic Index (HSI, %) = (liver weight (g) /whole body weight (g)) × 100¸ and Viscerosomatic Index (VSI, %) = (visceral weight (g) /whole body weight (g)) × 100.

2.6. Proximal composition and fatty acid profile.

Chemical analysis of feeds and carcasses was carried out following the methods by AOAC (2000) for dry matter and ash, whereas crude protein (N × 6.25) was determined by using elemental analysis (C:H:N) with a Fisons EA 1108 analyzer (Fisons Instruments, Beverly, MA, USA). Total lipid content was analysed following the procedures described by Folch (1957). Fatty acid profile of feeds and muscle samples was determined following the method of Rodríguez-Ruiz *et al*. (1998), using a gas chromatograph. For amino acid analysis, microalgal biomass and experimental diets were hydrolysed (20 mg in 1 mL HCl 6M) at 110ºC during 24 h under an inert atmosphere (N_2). After that, 50 µL of the hydrolysate were mixed with 50 µL of 6 M NaOH. Then 100 µL of internal standard (2.5 mM norleucine) and 800 µL sodium citrate loading buffer (pH 2.2) were added and mixed by vortex for 5 sec, and then filtered (0.2 µm). A sample (20 µL) of this mixture was analysed with an amino acid analyser Biochrom 30+ amino acid analyser (Biochrom LTD Cambridge, UK) according to the manufacturer's protocol.

2.7. Muscle lipid oxidation.

Lipid oxidation in muscle samples was estimated by measuring thiobarbituric acid-reactive substances (TBARS) following the method of Buege and Aust (1978). Samples (1 g) were homogenized in 4 mL 50 mM NaH_2PO_4, 0.1% (v/v) Triton X-100 solution. The mixture was centrifuged (10,000×*g*, 20 min, 4ºC). Supernatants were mixed in a 1:5 ratio (v/v) with 2-thiobarbituric acid (TBA) reagent (0.375% w/v TBA, 15% w/v TCA, 0.01% w/v 2,6- di-tert-butyl-4-methylphenol (BHT) and 0.25 N HCl), heated for 15 min and centrifuged (3,600×*g*, 10 min, 4ºC). The absorbance of supernatants was measured at 535 nm. The amount of TBARS was expressed as mg of malondialdehyde (MDA) per kg of muscle after comparing with a MDA standard.

2.8. Digestive enzyme activities.

Total alkaline protease activity was determined according to the method described by Alarcón *et al.* (1998) using buffered 5 g L^{-1} casein (50 mM Tris-HCl, pH 9.0) as substrate.

The amount of enzyme releasing 1 μg tyrosine per minute was defined as one unit of activity, considering an extinction coefficient for tyrosine of 0.008 μg^{-1} cm^{-1} mL^{-1}, measured spectrophotometrically at 280 nm. Trypsin and chymotrypsin activities were measured spectrophotometrically at 405 nm using 0.5 mM BAPNA (N-a-benzoyl-DL-arginine-4-nitroanilide) as substrate, according to Erlanger *et al*. (1961), and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-P-nitroanilide), following the method described by Del Mar *et al*. (1979), respectively. Leucine aminopeptidase activity was quantified using 2 mM L-leucine -p-nitroanilide (LpNa) in 100 mM Tris-HCl buffer (pH 8.8) as substrate according to Pfleiderer (1970). Alkaline phosphatase activity was determined using buffered p-nitrophenyl phosphate (pH 9.5) as substrate, following the method described by Bergmeyer (1974). For trypsin, chymotrypsin and leucine aminopeptidase activities, the amount of enzyme that released 1 μmol of p-nitroanilide (pNA) per minute was defined as one unit of activity, considering as extinction coefficient 8,800 M cm^{-1}, measured spectrophotometrically at 405 nm. For alkaline phosphatase, one unit of activity was defined as the amount of enzyme that released 1 μg of nitrophenyl per min (extinction coefficient 17,800 M cm^{-1} at 405 nm).

2.9. Ultrastructural analysis of intestinal mucosa.

Intestine samples from the anterior region were collected for evaluation by electron microscopy. Samples for scanning electron microscopy (SEM) were previously washed with 1% S-carboxymethyl-L-cysteine (Sigma) for 20 seconds in order to remove the epithelial mucus, and fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.2) for 24 h. Then, samples were washed and dehydrated in graded ethanol. Critical point drying of samples (CDP 030 Critical point dryer, Leica Microsystems, Madrid, Spain) was carried out by using absolute ethanol as intermediate fluid and CO_2 as transition fluid. After that, dried samples were mounted on supports and fixed with graphite (PELCO® Colloidal Graphite, Ted Pella INC., Ca, USA) and then gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). All samples were screened with a scanning

electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). Samples for transmission electron microscopy (TEM) were fixed in 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5 (4 h, 4 ºC). Then, intestine sections were washed with PBS for 20 min, subjected to a post-fixation step with 20 g L^{-1} osmium tetroxide, and dehydrated by consecutive immersion (20 min each) in ethanol solution of gradients from 50% to 100% (v/v). Samples were embedded for two hours in 1:1 Epon resin: absolute ethanol mixture under continuous shaking, and then included in pure Epon resin during 24 h, and polymerized at 60ºC. Finally, the ultrathin cuts were placed on 700Å copper mesh and stained with uranyl acetate and lead citrate. The mesh observation was performed with a Zeiss 10C transmission electron microscopy (Carl Zeiss, Barcelona, Spain) at 100 Kv. Visualization fields were recorded at ×16,000 magnification.

SEM and TEM visualization fields were recorded, and digital images were analysed using UTHSCSA ImageTool software. Microvilli length (ML) and microvilli diameter (MD) as well as the number of microvilli within 1µm distance (Vizcaíno *et al*., 2014) were determined in TEM micrographs. SEM images were used to obtain several measurements of enterocyte apical area (EA). Finally, data obtained from TEM and SEM images were used to estimate the total absorption surface per enterocyte (TAS) according to Vizcaíno *et al*. (2014).

2.10. Antimicrobial activities in liver homogenates.

Liver samples, stored at –80ºC, were homogenized (1 mg of tissue mL^{-1}) in phosphate buffer saline (PBS, pH 7,4) and protease, antiprotease, peroxidase, lysozyme and bactericidal activities were analysed in extracts. Protease activity was determined by measuring the hydrolysis of azocasein, according to the protocol by Charney and Tomarelly (1995) modified as described in Chaves-Pozo *et al*. (2019). Results were expressed as relative values, considering 100% protease activity that observed for a protease standard solution (10 µL of 2 mg mL^{-1} proteinase K (AppliChem) in PBS) under

the specified assay conditions. Results were then expressed as % μg^{-1} of tissue. The antiprotease activity was determined by the ability to inhibit proteinase K activity using a modification of the protocol described in Ellis (1990) as described elsewhere (Chaves-Pozo *et al*., 2019). The percentage of inhibition of proteinase K activity for each sample was calculated as 100 − (% of protease activity). Results were expressed as % μg^{-1} of tissue. Peroxidase activity was measured in extracts according to Quade *et al*. (1997) and Chaves-Pozo *et al*. (2019). One unit was defined as the amount of activity producing an absorbance change of 1, and the activity was expressed as units (U) μg^{-1} of tissue. The lysozyme was measured using a modification of the turbidimetric method described by Parry *et al*. (1965), using 0.3 mg mL^{-1} freeze-dried *Micrococcus lysodeikticus* (Sigma M-3770) in phosphate citrate buffer (0.13 M disodium phosphate, 0.11 M citrate and 0.015 M NaCl, pH 6.2) as substrate. The reduction in absorbance at 450 nm was measured immediately every 30 s during 15 min at 22 °C in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. Results were expressed as units (U) μg^{-1} of tissue. Bactericidal activity was determined by evaluating their effects on the bacterial growth of *Vibrio harveyi*, as explained elsewhere (Sunyer *et al*., 1995, Chaves-Pozo *et al*., 2019). Results were corrected with the absorbance measured in each sample at the initial time point and expressed as % μg^{-1} of tissue.

2.11. Statistical analysis.

Data were presented as mean ± SD. The effects of the different dietary treatments were determined by one-way ANOVA considering a significance level of 95% to indicate statistical difference ($P < 0.05$), followed by a generalized lineal statistical model (GLM analysis). Significant differences were determined using Tukey's multiple comparison test. Data with nonparametric distribution were analysed using Kruskal-Wallis one-way analysis. Also, discriminant analysis (DA) was conducted in digestive enzyme activities and morphometrical analysis of TEM and SEM images. The estimation of the validity of the discriminant function was based on the significance of Wilk's Lambda and the

percentage of correct assignment to a certain diet. All statistical analyses were performed with Statgraphics Plus 4.0 (Rockville, Maryland, USA) software.

3. Results.

3.1. Growth performance and proximate composition.

The evolution of fish growth during the feeding trial is shown in Fig. 1. Dietary inclusion of *A. platensis* biomass, either crude or hydrolysed, did not affect fish growth or nutrient utilization. In fact, final body weight (FBW), daily gain (DG), specific growth rate (SGR) and Fulton's body condition factor (K) were similar in all the experimental groups (Table 4). Regarding somatic indices, hepatosomatic index (HSI) tended to decrease in fish fed on hydrolysed *Arthrospira*-supplemented diets, although significant differences with respect to CT group were only observed for H-10 lot ($P < 0.05$). Viscerosomatic index (VSI) remained unaffected.

The proximate composition of eviscerated fish is shown in Table 5. The inclusion of *Arthrospira* biomass, crude or hydrolysed, did not modify protein, lipid, or ash contents of gilthead seabream fry ($P < 0.05$).

3.2. Fatty acid profile.

Muscle fatty acid composition of gilthead seabream fry is shown in Table 6. All the experimental groups showed similar saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid contents ($P > 0.05$). PUFAs were the predominant lipid fraction (32.68 – 33.42%), followed by MUFAs (30.77 – 31.18%) and SFAs (22.97 – 24.90%). Considered individually, muscle content of myristic acid (C14:0) and palmitic acid (C16:0) was significantly lower in fish fed on H-10 and H-5 diets, respectively, compared to the rest of dietary treatments evaluated. However, fish fed with crude *A. platensis*-supplemented diets (C-5 and C-10) showed the highest values of palmitic acid.

Regarding polyunsaturated fatty acids, the inclusion of microalgae hydrolysates (H-5 and H-10 diets) yielded a significant decrease in eicosapentaenoic acid (EPA, 20:5n3) content in comparison with CT group ($P < 0.05$), whereas docosahexaenoic acid (DHA, 22:6n3) tended to increase in these experimental groups, although no significant differences were observed. As a result, fish fed on diets containing microalgae hydrolysates (H-5 and H-10) showed lower EPA/DHA ratios ($P < 0.05$).

3.3. Muscle lipid oxidation (TBARS).

Results of muscle TBARS are shown in Fig. 2. Overall, fish fed on microalgae-supplemented diets showed a significant decrease in TBARS values compared to CT group ($P < 0.05$), especially in C-10, H-5 and H-10 experimental groups.

3.4. Digestive functionality.

Intestinal enzyme activities were measured at two time points throughout the feeding trial (Fig. 3). In general, the results obtained revealed that the dietary inclusion of crude and hydrolysed *A. platensis* had no adverse impact on the digestive enzymes evaluated, regardless of the dietary inclusion considered. Overall, the activity level of the different digestive enzymes evaluated increased significantly with larval age regardless of dietary treatment used ($P < 0.001$) (Table 7). At 75 dph, an increase of trypsin and chymotrypsin activity levels was found in groups fed with crude and hydrolysed microalgae compared to control (CT) ($P > 0.001$). Moreover, results showed that the use of the hydrolysates (H-5 and H-10) increased the chymotrypsin and total alkaline protease activity levels compared to fish fed with crude microalgal biomass (chymotrypsin, $P = 0.001$; total alkaline protease, $P = 0.012$). Regarding brush border enzymes, the inclusion of the microalgae biomass increased significantly both leucine aminopeptidase ($P = 0.015$) and alkaline phosphatase ($P = 0.004$) activities. Fish fed on C-5 and H-10 diets showed higher leucine aminopeptidase activity than CT group. For alkaline phosphatase, all the experimental groups fed on *A. platensis* supplemented diets, especially H-10 group,

displayed higher activity than fish receiving CT diet. At the end of the feeding trial (95 dph)only chymotrypsin, and leucine aminopeptidase activities increased owing to the inclusion of the microalga. C-10 and H-10 showed higher chymotrypsin activity levels than those observed in the control fish, whereas the increase of leucine aminopeptidase activity ($P < 0.05$) was observed in most groups feed on microalgae-supplemented diets, except C-10 group.

On the other hand, TEM and SEM images showed an intestinal mucosa with normal appearance in all the fish fed with the experimental diets. In general, a regular and densely packed microvilli on the enterocyte surface was observed in all the specimens without any sign of structural damage or microvilli alterations (Figs. 4A and 4B).

Morphometric analysis of TEM and SEM images revealed that the dietary inclusion of both crude and hydrolysed *A. platensis* caused a significant effect on microvilli length, microvilli diameter, enterocyte apical area and absorption surface ($P < 0.001$) (Table 8). Overall, fish fed on microalgae-supplemented diets showed significantly increased microvilli length and microvilli diameter compared to fish of CT group, not least owing to the use of the microalgal hydrolysate ($P < 0.001$), regardless the dietary inclusion level. In addition, H-10 fed fish showed the highest values of enterocyte apical area (EA) and enterocyte absorption surface (TAS).

Values of digestive enzyme activities and ultrastructural analysis of intestinal mucosa were analysed by discriminant analysis (DA) (Fig. 5, Table 9). Grouping was recognized on the basis of the diet ingested by fish. DA confirmed that function 1 could discriminate clearly diet H-10, which was plotted much further to the right than the other dietary treatments. The second function provided certain discrimination between the CT diet and the microalgae-supplemented diets, but not nearly as clear as that provided by the first function.

3.5. Immune system activities.

The immune activities analysed in liver extracts did not show significant differences between treatments ($P > 0.05$) (Table 10). These results indicate that the microalgae-supplemented diets did not cause any negative impact on the innate immune activities in the liver of gilthead seabream fry. On the contrary, protease activity tended to increase in fish fed on C-5 and H-10 diets, and lysozyme and bactericidal activities tended to increase in H-10 diet, although these changes were only significant for lysozyme activity (H-10 group), likely owing to the great variability observed between specimens.

4. Discussion.

The dietary inclusion of microalgae hydrolysates emerges as a novel strategy in aquaculture nutrition. The enzymatic hydrolysis of microalgae biomass may increase extraction yields of high-value products or improve the bioavailability of intracellular components (Galafat *et al*., 2020; Siddik *et al*., 2021), and therefore an improvement on fish growth parameters derived from its dietary inclusion could be expected. However, the results obtained in this work revealed that the dietary inclusion of crude and hydrolysed *A. platensis* biomass had no significant effect on body growth parameters after a 40-day feeding trial. These results are in agreement with those reported by Galafat *et al*. (2020), who pointed out similar effects in gilthead seabream juveniles fed on *Arthrospira* sp. protein hydrolysate up to 4% inclusion level. Similarly, Ayala *et al*. (2020) did not observe differences in growth rates and feed intake in gilthead seabream juveniles attributable to the dietary inclusion of *N. gaditana* either raw or hydrolysed. On the contrary, other authors reported that low dietary inclusion of different microalgae species, such as *Tetraselmis suecica, Phaeodactylum* sp., *Nannochloropsis* sp., or *Chlorella* sp., in microdiets led to improved growth performance in gilthead seabream and Senegalese sole fry (Vizcaíno *et al*., 2016; Peixoto *et al*., 2021). These disparate results confirm that microalgae have a species-specific and dose-dependent effect on growth performance and nutrient utilization of farmed fish.

Regarding somatic indices, the dietary inclusion of hydrolysed *A. platensis* decreased hepatosomatic index (HSI), especially with the highest inclusion level. This reduction of HSI has been previously documented in several studies (Vizcaíno *et al*., 2016; Tulli *et al*., 2012), and seems to be related to the existence of a direct relationship with lipid metabolism. Thus, some microalgae compounds are apparently able to modulate to a certain extent lipid metabolism by increasing the efficiency of lipid mobilization from liver to muscle, especially in phases of rapid growth (Knutsen *et al*., 2019). However, the results obtained in this work indicate that the dietary inclusion of crude or hydrolysed *A. platensis* did not modify gilthead seabream fry body composition, in line with earlier reports on juveniles of the same (Galafat *et al*., 2020), and other farmed fish species (Sorensen *et al*., 2017; Valente *et al*., 2019). The influence of diets on fish body composition can also be assessed by means of the muscle fatty acid profile (Fernandes *et al*., 2012). According to previous studies (Tibaldi *et al*. 2015; Cardinaletti *et al*., 2018; He *et al*., 2018), certain degree selective retention of specific fatty acids in the muscle of fish fed with microalgae-supplemented diets has been observed, especially EPA and DHA. Thus, muscle EPA contents were lower in fish fed on microalgae hydrolysate diets (H-5 and H-10) than those measured in the respective experimental diets, a fact that seems to be related to the modulating effect on lipid metabolism previously mentioned and with a higher catabolic use of this specific fatty acid (Tocher, 2003). Both EPA and DHA play a key role in fish development, and their dietary deficiency is related to adverse effects on fish growth and survival (Glencross, 2009; Tocher *et al*., 2010). In this work, EPA/DHA ratios in all the experimental diets ranged from 0.66 to 0.69, similar to the optimum (0.67) for gilthead seabream (Rodriguez *et al*., 1997), although this factor did not affect growth performance and nutrient utilization at the end of the feeding trial, as it has been observed in previous works (Vizcaíno *et al*., 2016; Atalah *et al*., 2007).

On the other hand, a significant reduction in MDA levels was observed in muscle of fry fed the microalgae-supplemented diets. This phenomenon might well be directly related

to enhanced scavenging activity against reactive oxygen species, resulting in lower muscle lipid oxidation (Teimouri *et al*., 2019). Microalgae contain a wide range of bioactive compounds like astaxanthin and xanthophylls able to prevent oxidative stress, as previously observed in fish fed on diets supplemented not only with *Arthrospira* sp. (Abdelkhalek *et al*., 2015; Teimouri *et al*., 2019; Galafat *et al*. 2020), but also with other microalgae species (Qiao *et al*., 2019; Batista *et al*., 2020). It is worth mentioning that low dietary inclusion of *Arthrospira* hydrolysate (H-5) caused a notable reduction in muscle MDA level; in fact, similar to that observed in fish fed with 10% crude microalgal biomass. This could be explained by the fact that enzymatic hydrolysis might have facilitated the release of antioxidant compounds that otherwise are less available within the intact cells (Liu *et al*., 2019; Afifi *et al*., 2018).

In addition to the effects on growth and fish chemical composition, the nutritional value of first-feeding diets, combined with other biotic and abiotic factors, play a key role on the ontogeny of the digestive tract in marine fish larvae (Yufera *et al*., 2000; Zambonino-Infante and Cahu, 2001). Indeed, imbalanced diets can lead to a disruption in maturation processes, such as a reduction in pancreatic secretory capacity (Zambonino-Infante *et al*., 2008). The dietary inclusion of microalgae and cyanobacteria has been previously evaluated in different farmed fish species, and roughly, no detrimental effects have been described on proteolytic enzyme activity (Vizcaíno *et al*., 2016; Gong *et al*., 2019; Galafat *et al*., 2020). In this work, partial replacement of fish meal with crude and hydrolysed *A. platensis* did not impair the pancreatic secretion of proteolytic enzymes, quite the contrary, given that fish fed on microalgae-supplemented diets showed higher trypsin and chymotrypsin activity levels than fish fed a fishmeal:fish oil (FM:FO) diet, especially in the case of fish fed with the microalgae hydrolysate. Both enzymes play a key role in early life stages, since digestion relies mainly on these pancreatic enzymes until a gastric digestion was attained (Hamre *et al*., 2013; Khoa *et al*., 2019). These enzymes are modulated by dietary protein content, so that the increase in activity observed could be

related to an increase in the availability of peptides and amino acids, which may stimulate the pancreatic secretion (Zambonin-Infante *et al*., 2008; Hamre *et al*., 2013). Indeed, Gisbert *et al*. (2018) evidenced differences in chymotrypsin activity in fish related to differences in the distribution of peptide molecular weight among hydrolysates obtained from fish, yeast and pig blood. Regarding brush border enzymes, our results revealed a lack of negative effects on leucine aminopeptidase and alkaline phosphatase activities in fish fed on *A. platensis* supplemented feeds. Fish from these experimental groups, especially H-5 and H-10, showed significantly increased activities for both enzymes, which seems to be related to adequate maturation of the intestine (Zambonino-Infante and Cahu 2001).

Despite this, the improvement in fish digestive enzyme activities did not translate into increased growth performance. This finding might be explained by two different hypotheses; on the one hand, the increase in enzyme activity levels could be associated with a compensation mechanism against dietary changes (Santigosa *et al*. 2008); and on the other, it could be related to a saturation of the peptide transport system in the intestinal brush-border membrane. In fact, the use of protein hydrolysates may induce an overload of short peptides, and/or impaired utilization of the small peptides and free amino acids, which are used for energy production rather than for growth (Yúfera and Moyano, 2018).

In addition to the effects on the digestive enzyme activities, inadequate dietary and environmental conditions may interfere with the complex regulation mechanisms involved in epithelial development, disrupting epithelial integrity (Roonestad *et al*., 2013). According to previous studies, the dietary inclusion of different species of microalgae exerts positive effects on the apical brush border integrity, such as increased microvilli length, enterocyte apical area, and increased absorption surface in enterocytes (Galafat *et al*., 2020; Vizcaíno *et al*., 2016; Araújo *et al*., 2016). In line with these studies, the results obtained revealed that the inclusion of *A. platensis* biomass caused favourable

effects on gut morphology of gilthead seabream fry, especially in the case of the hydrolysed microalgae. Hence, changes observed in microvilli length and microvilli diameter might improve the contribution of the intestinal mucosa as a natural barrier, whereas increased total absorption surface observed in fish fed on 10% hydrolysed biomass might enhance the absorptive capacity of the intestinal mucosa (Vizcaíno *et al*., 2014).

Regarding the innate immune system, it has been reported that the dietary inclusion of crude microalgae could modulate slightly protease activity, peroxidase and antibacterial activities, but not lysozyme activity (Chaves-Pozo *et al*., 2014), while stimulated cellular immune activities were reported in some cases (Cerezuela *et al.*, 2012). In this study, no detrimental effects on the immune status of gilthead seabream larvae could be attributed to *A. platensis*-supplemented diets, but on the contrary, fish fed on 10% hydrolysed microalgae showed increased lysozyme activity levels, this fact suggesting better defence against infectious diseases. However, the discrepancies observed among different studies assessing the impact of microalgae on the activity of the innate immune system suggest both a species-specific and dose-specific effect on the results reported.

In conclusion, the results obtained in this study indicate that *A. platensis* hydrolysate can be used as dietary ingredient in starter feeds for gilthead seabream fry. The use of enzymatically hydrolysed microalgae up to 10% inclusion level did not improve body growth parameters, but yielded positive effects on gut functionality, muscle lipid peroxidation, and lysozyme activity in liver, which seems to be associated with an increase in the nutritional and functional properties of this biomass compared with the raw microalgae. These positive effects confirm that this supplement can be included as a functional ingredient in starter feeds for gilthead seabream.

Authors' contributions.

Alarcón, F.J. and Martínez T.F. conceived and designed the experiments and prepared the aquafeeds. Galafat, A., Vizcaíno, A.J., and Sáez, M.I., performed the fish sampling. Arizcun, M. and Chaves-Pozo, E., participated in sampling, fish maintenance and immune activities analysis. Galafat, A., Vizcaíno, A.J., Sáez, M.I., Martínez, T.F., Arizcun M., and Chaves-Pozo E., performed analytical analysis and discussed the data. Galafat, A., Vizcaíno, A.J., and Alarcón, F.J., performed the data analysis and drafted the manuscript. All authors critically revised and approved the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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 the Instituto Español de Oceanografía (REGA code ES300261040017).

References.

Abdelkhalek, N.K., Ghazy, E.W., Abdel-Daim, M.M., 2015. Pharmacodynamic interaction of *Spirulina platensis* and deltamethrin in freshwater fish Nile tilapia, *Oreochromis niloticus*: impact on lipid peroxidation and oxidative stress. Environ. Sci. Pollut. Res., 22(4), 3023-3031. http://doi.org/10.1007/s11356-014-3578-0

Afify, A.E.M.M., El Baroty, G.S., El Baz, F.K., Abd El Baky, H.H., Murad, S.A., 2018. *Scenedesmus obliquus*: Antioxidant and antiviral activity of proteins hydrolysed by three enzymes. J. Genet. Eng. Biotechnol., 16(2), 399-408. http://doi.org/10.1016/j.jgeb.2018.01.002

Alarcón, F.J., Díaz, M., Moyano, F.J., Abellán, E., 1998. Characterization and functional properties of digestive proteases in two sparid; gilthead sea bream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiol. Biochem., 19, 257-267. http://doi.org/10.1023/A:1007717708491

Araújo, M., Rema, P., Sousa-Pinto, I., Cunha, L.M., Peixoto, M.J., Pires, M.A., Seixas, F., Brotas, V., Beltrán, C., Valente, L., 2016. 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. 28:679–689. http://doi.org/10.1007/s10811-015-0591-8

Atalah, E., Cruz, C.H., Izquierdo, M.S., Rosenlund, G., Caballero, M.J., Valencia, A., Robaina, L., 2007. Two microalgae *Crypthecodinium cohnii* and *Phaeodactylum tricornutum* as alternative source of essential fatty acids in starter feeds for

seabream (*Sparus aurata*). Aquaculture, 270(1-4), 178-185. http://doi.org/10.1016/j.aquaculture.2007.04.009

Ayala, M.D., Galián, C., Fernández, V., Chaves-Pozo, E., García de la Serrana, D., Sáez, M.I., Galafat A., Alarcón F.J., Martínez, T.F., Arizcun, M., 2020. Influence of low dietary inclusion of the microalga *Nannochloropsis gaditana* (Lubián 1982) on performance, fish morphology, and muscle growth in juvenile gilthead seabream (*Sparus aurata*). Animals, 10(12), 2270. http://doi.org/10.3390/ani10122270

Batista, S., Pereira, R., Oliveira, B., Baião, L.F., Jessen, F., Tulli, F., Messina, M., Silva, J., Abreu, H., Valente, L.M., 2020. Exploring the potential of seaweed *Gracilaria gracilis* and microalga *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for European seabass *Dicentrarchus labrax*. J. Appl. Phycol., 32, 2041-2059. http://doi.org/10.1007/s10811-020-02118-z

Bergmeyer, H.V., 1974. Phosphatases methods of enzymatic analysis, Vol. 2. Phosphatases, Academic Press, New York, pp. 856-860.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. In Methods in enzymology (Vol. 52, pp. 302-310). Academic Press.

Cahu, C., Infante, J.Z., 2001. Substitution of live food by formulated diets in marine fish larvae. Aquaculture. 200 (1-2), 161-180. http://doi.org/10.1016/S0044-8486(01)00699-8

Cai, Z., Li, W., Mai, K., Xu, W., Zhang, Y., Ai, Q., 2015. Effects of dietary size-fractionated fish hydrolysates on growth, activities of digestive enzymes and aminotransferases and expression of some protein metabolism related genes in large yellow croaker (*Larimichthys crocea*) larvae. Aquaculture. 440, 40-47. http://doi.org/10.1016/j.aquaculture.2015.01.026

Cardinaletti, G., Messina, M., Bruno, M., Tulli, F., Poli, B. M., Giorgi, G., Chini-Zittelli, G., Tredici, M., Tibaldi, E., 2018. Effects of graded levels of a blend of *Tisochrysis lutea* and *Tetraselmis suecica* dried biomass on growth and muscle tissue composition of European sea bass (*Dicentrarchus labrax*) fed diets low in fish meal and oil. Aquaculture, 485, 173-182. http://doi.org/10.1016/j.aquaculture.2017.11.049

Cerezuela, R., FA. Guardiola, P. Gonzalez, J. Meseguer, MA. Esteban., 2012. Effects of dietary *Bacillus subtilis, Tetraselmis chuii*, and *Phaeodactylum tricornutum*, singularly or in combination, on the immune response and disease resistance of sea bream (*Sparus aurata* L.). Fish Shellfish Immunology 33: 342-349. http://doi.org/10.1016/j.fsi.2012.05.004

Charney, J., Tomarelly, R.M., 1947. A colorimetric method for the determination of the proteolytic activity of duodenal juice. J. Biol. Chem. 171, 501–505. http://doi.org/10.1016/s0021-9258(17)41059-3

Chaves-Pozo, E., Abellán, E., Baixauli, P., Arizcun, M., 2019. An overview of the reproductive cycle of cultured specimens of a potential candidate for Mediterranean aquaculture, *Umbrina cirrosa*. Aquaculture 505, 137-149. http://doi.org/10.1016/j.aquaculture.2019.02.039

Chaves-Pozo, E., Arizcun, M., Vizcaíno, A., García-Cubero, R., Abellán, E., 2014. Effects of microalgae supplementation diets on the humoral immune response of gilthead seabream (*Sparus aurata*) larvae. The European Aquaculture Society at the Aquaculture Europe 14 Congress.

Conceição, L.E.C., Morais, S., Rønnestad, I., 2007. Tracers in fish larvae nutrition: A review of methods and applications. Aquaculture. 267 (1-4), 62-75. http://doi.org/10.1016/j.aquaculture.2007.02.035

Del Mar, E.G., Largman, C., Broderick, J.W., Geokas, M.C., 1979. A sensitive new substrate for chymotrypsin. Anal. Biochem., 99, 316-320. http://doi.org/10.1016/s0003-2697(79)80013-5

Ellis, A.E., 1990. Serum antiproteases in fish. In: Techniques in fish immunology. (eds. et al) 95-99. Fair Haven, N.J. SOS Publications.

Erlanger, B., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys., 95, 271-278. http://doi.org/10.1016/0003-9861(61)90145-X

Fernandes, T.J.R., Alves, R.C., Souza, T., Silva, J.M.G., Castro-Cunha, M., Valente, L.M.P., Oliveira, M.B.P.P., 2012. Lipid content and fatty acid profile of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles as affected by feed containing different amounts of plant protein sources. Food Chem. 134(3), 1337-1342. http://doi.org/10.1016/j.foodchem.2012.03.026

Folch, J., 1957. A simple method for the isolation and purification of total lipids form animal tissues. J. Biol. Chem., 226. 497-509. http://doi.org/10.1016/S0021-9258(18)64849-5

Galafat, A., Vizcaíno, A.J., Sáez, M.I., Martínez, T.F., Jerez-Cepa, I., Mancera, J.M., Alarcón, F.J., 2020. Evaluation of *Arthrospira sp*. enzyme hydrolysate as dietary additive in gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Phycol. 32: 3089–3100. http://doi.org/10.1007/s10811-020-02141-0

Gisbert, E., Fournier, V., Solovyev, M., Skalli, A., Andree, K.B., 2018. Diets containing shrimp protein hydrolysates provided protection to European sea bass (*Dicentrarchus labrax*) affected by a *Vibrio pelagius* natural infection outbreak. Aquaculture, 495, 136-143. http://doi.org/10.1016/j.aquaculture.2018.04.051

Glencross, B.D., 2009. Exploring the nutritional demand for essential fatty acids by aquaculture species. Rev. Aquacult., 1(2), 71-124. http://doi.org/10.1016/S0044-8486(01)00644-5

Gong Y., Bandara T., Huntley M., Johnson Z.I., Dias J., Dahle D., Sørensen M., Kiron V., 2019. Microalgae *Scenedesmus* sp. as a potential ingredient in low fishmeal diets for Atlantic salmon (*Salmo salar* L.). Aquaculture, 501: 455–464. http://doi.org/10.1016/j.aquaculture.2018.11.049

Guiry, M.D., Guiry, G.M., 2018. AlgaeBase. http://www.algaebase.org. Galway, Ireland: National University of Ireland. Accessed: 27 May 2021.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Rev. Aquacult., 5, S26-S58. http://doi.org/10.1111/j.1753-5131.2012.01086.x

Han, P., Lu, Q., Fan, L., Zhou, W., 2019. A review on the use of microalgae for sustainable aquaculture. Appl. Sci., 9(11), 2377. http://doi.org/10.3390/app9112377

He, Y., Lin, G., Rao, X., Chen, L., Jian, H., Wang, M., Guo, Z., Chen, B., 2018. Microalga *Isochrysis galbana* in feed for *Trachinotus ovatus*: effect on growth performance and fatty acid composition of fish fillet and liver. Aquacult. Int., 26(5), 1261-1280. http://doi.org/10.1007/s10499-018-0282-y

Khoa, T.N.D., Waqalevu, V., Honda, A., Shiozaki, K., Kotani, T., 2019. Early ontogenic development, digestive enzymatic activity and gene expression in red sea bream (*Pargus major*). Aquaculture. 512, 734283. http://doi.org/10.1016/j.aquaculture.2019.734283

Knutsen, H.R., Johnsen, I.H., Keizer, S., Sørensen, M., Roques, J.A.C., Heden, I., Sundel K., Hagen, Ø., 2019. Fish welfare, fast muscle cellularity, fatty acid and body-composition of juvenile spotted wolffish (*Anarhichas minor*) fed a combination of plant proteins and microalgae (*Nannochloropsis oceanica*). Aquaculture, 506, 212-223. http://doi.org/10.1016/j.aquaculture.2019.03.043

Liu, C., Liu, H., Xu, W., Han, D., Xie, S., Jin, J., Yang, Y., Zhu, X., 2019. Effects of dietary *Arthrospira platensis* supplementation on the growth, pigmentation, and antioxidant in yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 510, 267-275. http://doi.org/10.1016/j.aquaculture.2019.05.067

Mahmoud, M.M., El-Lamie, M.M., Kilany, O.E., Dessouki, A.A., 2018. *Spirulina* (*Arthrospira platensis*) supplementation improves growth performance, feed utilization, immune response, and relieves oxidative stress in Nile tilapia (*Oreochromis niloticus*) challenged with *Pseudomonas fluorescens*. Fish. Shellfish. Immunol*.,* 72, 291-300. http://doi.org/10.1016/j.fsi.2017.11.006

Niccolai, A., Zittelli, G.C., Rodolfi, L., Biondi, N., Tredici, M.R., 2019. Microalgae of interest as food source: biochemical composition and digestibility. Algal res., 42, 101617. http://doi.org/10.1016/j.algal.2019.101617

Novak, M., Vetvicka, V., 2009. Glucans as Biological Response Modifiers. Endocrine, Metab. Immune Disord. - Drug Targets 9, 67-75. http://doi.org/10.2174/187153009787582423

Parry Jr, R.M., Chandan, R.C., Shahani, K.M., 1965. A rapid and sensitive assay of muramidase. Procedings Soc. Exp. Biol. Med., 119(2), 384-386. https://doi.org/10.3181/00379727-119-30188.

Peixoto, D., Pinto, W., Gonçalves, A. T., Machado, M., Reis, B., Silva, J., Navalho, J., Días, J., Conceição, L., Costas, B., 2021. Microalgal biomasses have potential as

ingredients in microdiets for Senegalese sole (*Solea senegalensis*) post-larvae. J. Appl. Phycol., 1-10. http://doi.org/10.1007/S10811-021-02431-1

Pfleiderer, G., 1970. Particle-bound aminopeptidase from pig kidney. Methods Enzymol 19:514-521. http://doi.org/10.1016/0076-6879(70)19038-0

Qiao, H., Hu, D., Ma, J., Wang, X., Wu, H., Wang, J., (2019). Feeding effects of the microalga *Nannochloropsis* sp. on juvenile turbot (*Scophthalmus maximus* L.). Algal Research, 41, 101540. http://doi.org/10.1016/j.algal.2019.101540

Quade, M.J., Roth, J.A., 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet. Immunol. Immunopathol. 58, 239-248. http://doi.org/10.1016/s0165-2427(97)00048-2

Rajasekar, P., Palanisamy, S., Anjali, R., Vinosha, M., Elakkiya, M., Marudhupandi, T., Tabarsa, M., You, S., Prabhu, N.M., 2019. Isolation and structural characterization of sulphated polysaccharide from Spirulina platensis and its bioactive potential: *In vitro* antioxidant, antibacterial activity and Zebrafish growth and reproductive performance. Int. J. Biol. Macromol., 141, 809-821. http://doi.org/10.1016/j.ijbiomac.2019.09.024

Rodriguez, C., Pérez, J.A., Diaz, M., Izquierdo, M.S., Fernández-Palacios, H., Lorenzo, A., 1997. Influence of the EPADHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. Aquaculture, 150(1-2), 77-89. http://doi.org/10.1016/S0044-8486(96)01472-X.

Rodríguez-Ruiz, J., Belarbi, E.H., Sanchez, J.L.G., Alonso, D.L., 1998. Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. Biotechnology techniques, 12(9), 689-691. http://doi.org/10.1023/A:1008812904017

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev. AquaculT., 5, S59-S98. http://doi.org/10.1111/raq.12010

Roohani, A.M., Kenari, A.A., Kapoorchali, M.F., Borani, M.S., Zorriehzahra, M.J., Smiley, A.H., Esmaili, M., Rombenso, A.N., 2019. Effect of spirulina *Spirulina platensis* as a complementary ingredient to reduce dietary fishmeal on the growth performance, whole-body composition, fatty acid and amino acid profiles, and pigmentation of Caspian brown trout (*Salmo trutta caspius*) juveniles. Aquacult. Nut., 25, 633-645. http://doi.org/10.1111/anu.12885

Saadaoui, H., Espejo-Carpio, F.J., Guadix, E.M., Amar, R.B., Pérez-Gálvez, R., 2019. Bi-objective optimization of tuna protein hydrolysis to produce aquaculture feed ingredients. Food Bioprod. Proces., 115, 26-35. http://doi.org/10.1016/j.fbp.2019.03.001

Sahoo, P.K., Mukherjee, S.C., 2001. Effect of dietary β-1,3 glucan on immune responses and disease resistance of healthy and aflatoxin B1-induced immunocompromised rohu (*Labeo rohita* Hamilton). Fish Shellfish Immunol. 11, 683–695. http://doi.org/10.1006/fsim.2001.0345

Santigosa, E., Sánchez, J., Médale, F., Kaushik, S., Pérez-Sánchez, J., Gallardo, M.A., 2008. Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fishmeal replacement by plant protein sources. Aquaculture 282:68-74. http://doi.org/10.1016/j.aquaculture.2008.06.007

Savoie, A., Le François, N.R., Lamarre, S.G., Blier, P.U., Beaulieu, L., Cahu, C., 2011. Dietary protein hydrolysate and trypsin inhibitor effects on digestive capacities and performances during early-stages of spotted wolffish: Suggested mechanisms.

Comp. Biochem. Physiol. A, 158(4), 525-530. http://doi.org/10.1016/j.cbpa.2010.12.017

Shah, M.R., Lutzu, G.A., Alam, A., Sarker, P., Chowdhury, M.K., Parsaeimehr, A., Liang, Y., Daroch, M., 2018. Microalgae in aquafeeds for a sustainable aquaculture industry. J. Appl. Phycol., 30(1): 197–213. http://doi.org/10.1007/s10811-017-1234-z

Siddik, M.A., Howieson, J. Fotedar, R., Partridge, G.J., 2021. Enzymatic fish protein hydrolysates in finfish aquaculture: a review. Rev Aquac, 13(1), 406-430. https://doi.org/10.1111/raq.12481

Sørensen, M., Gong, Y., Bjarnason, F., Vasanth, G.K., Dahle, D., Huntley, M., Kiron, V., 2017. *Nannochloropsis oceania*-derived defatted meal as an alternative to fishmeal in Atlantic salmon feeds. PloS one, 12(7), e0179907. http://doi.org/10.1371/journal.pone.0179907

Srichanun, M., Tantikitti, C., Kortner, T.M., Krogdahl, Å., Chotikachinda, R., 2014. Effects of different protein hydrolysate products and levels on growth, survival rate, and digestive capacity in Asian seabass (*Lates calcarifer* Bloch) larvae. Aquaculture, 428, 195-202. http://doi.org/10.1016/j.aquaculture.2014.03.004

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, 333-345. http://doi.org/10.1016/0165-2427(94)05430-z

Teimouri, M., Yeganeh, S., Mianji, G.R., Najafi, M., Mahjoub, S., 2019. The effect of *Spirulina platensis* meal on antioxidant gene expression, total antioxidant capacity, and lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*). Fish Physiol. Biochem., 45(3), 977-986. http://doi.org/10.1007/s10695-019-0608-3

Tibaldi, E., Chini Zittelli, G., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., Venturini, S., Tredici, M.R., Poli, B.M., 2015. Growth performance and quality traits of European seabass (*D. labrax*) fed diets including increasing levels of freeze-dried *Isochrysis* sp. (T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. Aquaculture, 440, 60–68. http://doi.org/10.1016/j.aquaculture.2015.02.002

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish Sci. 11(2), 107-184. http://doi.org/10.1080/713610925

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquacult. Res., 41(5), 717-732. http://doi.org/10.1111/j.1365-2109.2008.02150.x

Torres, M., Navarro, J.C., Varó, I., Agullero, M.J., Morais, S., Monroig, Ó., Hontoria, F., 2020. Expression of genes related to long-chain (C18-22) and very long-chain (> C24) fatty acid biosynthesis in gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) larvae: Investigating early ontogeny and nutritional regulation. Aquaculture, 734949. http://doi.org/10.1016/j.aquaculture.2020.734949

Tulli, F., Chini Zittelli, G., Giorgi, G., Poli, B.M., Tibaldi, E., Tredici, M.R., 2012. Effect of the inclusion of dried *Tetraselmis suecica* on growth, feed utilization, and fillet composition of European sea bass juveniles fed organic diets. J. Aquat. Food Prod. Technol., 21, 188–197. http://doi.org/10.1080/10498850.2012.664803

Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in teleost fish: a review. Vet. Med. (Praha). 56, 486–503. http://doi.org/10.17221/3294-VETMED

Valente L.M.P., Custódio, M., Batista, S., Fernandes, H., Kiron, V., 2019. Defatted microalgae (*Nannochloropsis* sp.) from biorefinery as a potential feed protein

source to replace fishmeal in European sea bass diets. Fish Physiol. Biochem., 45(3), 1067-1081. http://doi.org/10.1007/s10695-019-00621-w

Vizcaíno A.J., López G., Sáez M.I., Jiménez J.A., Barros A., Hidalgo L., Camacho-Rodríguez J., Martínez T.F., Cerón-García M.C., Alarcón F.J., 2014. Effects of the microalga *Scenedesmus almeriensis* as fishmeal alternative in diets for gilthead sea bream, *Sparus aurata*, juveniles. Aquaculture, 431, 34–43. http://doi.org/10.1016/j.aquaculture.2014.05.010

Vizcaíno A.J., Saéz M.I., López G., Arizcun M., Abellán E., Martínez T.F., Alarcón F.J., 2016. *Tetraselmis suecia* and *Tisochrysis lutea* meal as dietary ingredients for gilthead seabream (*Sparus aurata* L.) fry. J. Appl. Phycol., 28(5), 2843-2855. http://doi.org/10.1007/s10811-016-0845-0

Wu, N., Song, Y.L., Wang, B., Zhang, X.Y., Zhang, X.J., Wang, Y.L., Cheng, Y.Y., Chen, D.D., Xia, X.Q., Lu, Y.S., Zhang, Y.A., 2016. Fish gut-liver immunity during homeostasis or inflammation revealed by integrative transcriptome and proteome studies. Sci. Rep. 6, 36048. http://doi.org/10.1038/srep3604

Siddik, M.A., Howieson, J. Fotedar, R., Partridge, G.J., 2021. Enzymatic fish protein hydrolysates in finfish aquaculture: a review. Rev Aquac, 13(1), 406-430. https://doi.org/10.1111/raq.12481

Yúfera M., Fernández-Diaz C., Pascual E., Sarasquete C., Moyano F.J., Diaz M., Alarcon F.J., Garcia-Gallego M., Parra G., 2000. Towards an inert diet for first-feeding gilthead seabream (*Sparus aurata* L) larvae. Aquacult. Nutr., 6, 143-152. http://doi.org/10.1046/j.1365-2095.2000.00110.x

Yúfera, M., Moyano, F.J., Martínez-Rodríguez, G., 2018. The digestive function in developing fish larvae and fry. From molecular gene expression to enzymatic

activity. In: Emerging Issues in Fish Larvae Research. http://doi.org/10.1007/978-3-319-73244-2_3

Zambonino-Infante J.L., Cahu C.L., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comp. Biochem. Physiol., 130C, 477-487. http://doi.org/10.1016/S1532-0456(01)00274-5

Zambonino-Infante, J.L., Gisbert, E., Sarasquete, S., Navarro, I., Gutiérrez, J., Cahu, C.L., 2008. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino JEP, Bureau D, Kapoor BG (eds) Feeding and Digestive Functions in Fishes, pp. 281–348. Science Publishers, Enfield, NH.

Tables

Table 1. Ingredients and proximate composition (g kg^{-1} DW) of the experimental diets.

	CT	C-5	C-10	H-5	H-10
Ingredients					
Fishmeal LT94[1]	685.0	641.0	597.0	641.0	597.0
A. platensis meal[2]		50.0	100.0		
A. platensis hydrolysate[3]				50.0	100.0
Attractant premix[4]	80.0	80.0	80.0	80.0	80.0
Wheat gluten[5]	50.0	50.0	50.0	50.0	50.0
Soybean protein concentrate[6]	20.0	20.0	20.0	20.0	20.0
Fish oil	53.0	55.0	58.0	55.0	58.0
Soybean lecithin[7]	40.0	40.0	40.0	40.0	40.0
Choline chloride	2.0	2.0	2.0	2.0	2.0
Wheat meal[8]	17.0	9.0		9.0	
Betaine	2.0	2.0	2.0	2.0	2.0
Vitamins and minerals premix[9]	30.0	30.0	30.0	30.0	30.0
Vitamin C	1.0	1.0	1.0	1.0	1.0
Binder (alginate)	20.0	20.0	20..0	20..0	20.0
Proximate composition (% DW)					
Crude protein	58.9	59.1	59.3	59.3	59.4
Crude lipid	18.1	18.0	17.9	18.4	18.3
Ash	16.0	16.0	15.7	15.8	16.1
Crude fiber	2.1	1.9	2.3	2.0	2.2
NfE[10]	4.9	5.0	4.8	4.5	4.0

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. [1](protein: 69.4%; lipid: 12.3%), Norsildemel (Bergen, Norway); [2](protein: 60.5%; lipid: 5.6%); [3]Liquid product containing 150 g microalgae meal L^{-1}; [4](50% squid meal, 25% shrimp meal, 25% krill meal); [5](protein: 76.0%; lipid: 1.9%); [6](protein: 50.0%; lipid: 1.0%); [7]Lecico P700 IP (Lecico GmbH, Germany) [8](protein: 12.0%; lipid: 2.0%); [9]Vitamin & Mineral Premix: Vitamins (IU or mg kg^{-1} premix): vitamin A (retinyl acetate), 2,000,000 IU; vitamin

D3 (DL-cholecalciferol), 200,000 IU; vitamin E , 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2,500 mg; vitamin B1(thiamine hydrochloride), 3,000 mg; vitamin B2 (riboflavin), 3,000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2,000 mg; vitamin B9 (folic acid), 1,500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (Lifebioencapsulation SL, Almería Spain); [10]NfE: Nitrogen free extract calculated as 100 – (% crude protein + % ether extract + % ash + % crude fiber). DW: dry weight.

Table 2. Fatty acid composition (% DW of relevant fatty acids) of the experimental diets.

	CT	C-5	C-10	H-5	H-10
14:0	3.0	3.0	3.1	2.8	2.9
16:0	16.0	16.8	16.5	16.8	16.4
18:0	2.8	2.7	2.7	2.7	2.8
18:1n9	19.0	19.2	19.3	19.5	19.3
18:2n6	12.6	12.9	12.7	13.4	13.0
18:3n3	2.0	2.0	1.9	1.8	1.9
20:4n6, ARA	0.9	0.9	0.9	0.9	0.9
20:5n3, EPA	7.5	7.3	7.2	6.8	7.1
22:6n3, DHA	10.8	10.6	10.4	10.3	10.5
SFA	21.9	22.5	22.2	22.3	22.1
HUFA	21.9	21.4	21.6	20.9	21.4
n3	24.5	23.8	23.8	23.0	23.7
n6	13.4	13.8	14.1	14.3	13.9
n9	5.9	5.7	5.8	5.4	5.6

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. DW: dry weight.

Table 3. Aminoacids profile of *A. platensis* biomass and the experimental diets (g 100 g^{-1} DW).

	A. platensis	**CT**	**C-5**	**C-10**	**H-5**	**H-10**
NEAA						
Ala	5.0	3.6	3.2	3.7	3.3	3.6
Asp	6.5	5.3	4.9	4.7	4.9	4.8
Cys	0.3	0.3	0.2	0.3	0.2	0.3
Glu	8.9	8.9	7.9	8.8	8.0	8.5
Gly	3.3	3.6	3.2	3.5	3.2	3.4
Pro	4.9	5.1	4.5	4.7	4.5	4.8
Ser	3.4	2.6	2.4	2.6	2.4	2.5
Tyr	3.2	2.0	1.9	1.9	1.9	2.0
EAA						
Arg	4.1	3.4	3.2	3.5	3.2	3.4
His	1.2	1.3	1.3	1.2	1.2	1.3
Ile	3.1	2.1	2.0	2.4	2.0	2.3
Leu	5.1	4.0	3.8	4.1	3.8	4.1
Lys	4.2	5.6	5.2	5.4	5.1	5.4
Met	0.9	1.6	1.4	1.5	1.4	1.5
Phe	3.6	2.6	2.4	2.5	2.4	2.6
Thr	3.3	2.4	2.1	2.3	2.2	2.2
Val	3.4	2.5	2.3	2.8	2.4	2.7
EAA/NEAA	0.9	0.8	0.8	0.9	0.8	0.9

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. EAA: essential aminoacids, NEAA: non-essential aminoacids. DW: dry weight

Table 4. Growth performance and nutrient utilization parameters of gilthead seabream fry fed with the experimental diets for 40 days.

	CT	C-5	C-10	H-5	H-10	*p-value*
Growth performance						
Final body weight (FBW, mg)	749.78 ± 126.28	714.23 ± 196.81	795.19 ± 173.62	766.79 ± 190.87	723.78 ± 190.95	*0.276*
Fulton's condition factor (K)	1.41 ± 0.12	1.36 ± 0.08	1.37 ± 0.03	1.48 ± 0.07	1.34 ± 0.04	*0.300*
Daily gain (DG, mg day^{-1})	19.93 ± 2.94	16.10 ± 4.58	17.98 ± 4.04	17.31 ± 4.44	16.32 ± 0.44	*0.277*
Specific Growth Rate (SGR, %/d)	8.17± 0.40	8.01 ± 0.65	8.29 ± 0.53	8.19 ± 0.59	8.05 ± 0.62	*0.215*
Somatic index						
Hepatosomatic Index (HSI, %)	2.42 ± 0.42 b	2.29 ± 0.61 b	2.10 ± 0.63 b	1.95 ± 0.65 ab	1.77 ± 0.36 a	*<0.001*
Viscerosomatic Index (VSI, %)	6.30 ± 1.20	6.39 ± 1.12	6.23 ± 1.06	5.82 ± 1.00	6.22 ± 1.27	*0.296*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate tanks (n=3). Values in the same row with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

Table 5. Carcass proximate composition (% of DW) of gilthead seabream at the end of the feeding trial.

	Protein	**Lipid**	**Ash**
CT	66.17 ± 0.16	15.24 ± 0.27	3.76 ± 0.02
C-5	65.81 ± 1.54	15.46 ± 0.28	3.65 ± 0.08
C-10	67.17 ± 0.04	15.23 ± 0.19	3.67 ± 0.21
H-5	65.08 ± 0.12	15.43 ± 0.37	3.63 ± 0.05
H-10	67.39 ± 0.02	15.53 ± 0.48	3.59 ± 0.04
P-value	*0.0882*	*0.7804*	*0.2192*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate determination. DW: dry weight.

Table 6. Muscle fatty acid composition (% fatty acids) of gilthead seabream fry fed with the experimental diets for 40 days.

	CT	C-5	C-10	H-5	H-10	*P-value*
14:0	2.40 ± 0.07 b	2.39 ± 0.04 b	2.40 ± 0.07 b	2.33 ± 0.09 ab	2.14 ± 0.01 a	*0.029*
16:0	16.86 ± 0.12 b	17.25 ± 0.05 c	17.59 ± 0.26 c	16.27 ± 0.14 a	16.71 ± 0.01 b	*0.002*
18:0	4.99 ± 0.02	4.89 ± 0.14	4.90 ± 0.06	4.37 ± 0.02	4.57 ± 0.02	*0.594*
16:1n7	4.59 ± 0.08	4.75 ± 0.18	4.78 ± 0.11	4.89 ± 0.04	4.87 ± 0.02	*0.153*
18:1n9	17.82 ± 0.07	17.97 ± 0.16	18.24 ± 0.32	12.21 ± 0.12	18.16 ± 0.15	*0.254*
18:1n7	3.26 ± 0.03	3.29 ± 0.03	3.25 ± 0.07	3.26 ± 0.01	3.24 ± 0.01	*0.729*
18:2n6	10.81 ± 0.01 a	11.32 ± 0.17 b	11.70 ± 0.22 b	11.63 ± 0.06 b	11.72 ± 0.03 b	*0.003*
18:3n3	1.44 ± 0.01	1.50 ± 0.02	1.53 ± 0.06	1.52 ± 0.02	1.45 ± 0.02	*0.087*
20:1n9	5.10 ± 0.06	4.88 ± 0.07	4.81 ± 0.31	4.82 ± 0.06	4.57 ± 0.19	*0.180*
20:4n6, ARA	0.46 ± 0.01	0.39 ± 0.01	0.42 ± 0.03	0.36 ± 0.02	0.38 ± 0.05	*0.069*
20:5n3, EPA	5.13 ± 0.13 c	5.22 ± 0.06 c	4.99 ± 0.01 c	3.81 ± 0.03 b	3.44 ± 0.04 a	*<0.001*
22:5n3	1.72 ± 0.04 b	1.70 ± 0.04 ab	1.66 ± 0.01 ab	1.60 ± 0.00 a	1.62 ± 0.01 a	*0.020*
22:6n3, DHA	12.61 ± 0.34 ab	12.11 ± 0.39 a	11.94 ± 0.22 a	12.45 ± 0.12 ab	13.21 ± 0.01 b	*0.026*
SFA	24.25 ± 0.17	24.53 ± 0.15	24.90 ± 0.39	22.97 ± 0.25	23.41 ± 0.04	*0.489*
MUFA	30.77 ± 0.07	30.89 ± 0.44	31.08 ± 0.81	31.18 ± 0.16	30.85 ± 0.01	*0.845*
PUFA	33.35 ± 0.51	33.41 ± 0.43	33.42 ± 0.01	32.68 ± 0.06	33.07 ± 0.12	*0.605*
Other FA	9.94 ± 0.29 ab	9.42 ± 0.08 ab	8.64 ± 0.92 a	11.43 ± 0.35 b	10.83 ± 0.17 b	*0.010*
n3	20.90 ± 0.51 b	20.53 ± 0.46 ab	20.12 ± 0.18 ab	19.39 ± 0.17 a	19.71 ± 0.06 ab	*0.029*
n6	11.27 ± 0.00 a	11.71 ± 0.18 ab	12.11 ± 0.25 b	11.99 ± 0.07 b	12.11 ± 0.02 b	*0.007*

n9	22.92 ± 0.12	22.85 ± 0.23	23.04 ± 0.63	23.04 ± 0.18	22.73 ± 0.05	*0.519*
n3/n6	1.85 ± 0.05	1.75 ± 0.07	1.66 ± 0.05	1.62 ± 0.02	1.63 ± 0.01	*0.595*
EPA/DHA	0.41 ± 0.00 c	0.43 ± 0.01 d	0.42 ± 0.01 cd	0.31 ± 0.00 b	0.26 ± 0.00 a	*<0.001*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate determination. Values in the same row with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

Table 7. Evaluation of different factors on digestive enzyme activities in the intestinal extracts of gilthead seabream at the end of the feeding trial.

	Age	Microalgae	Inclusion level	Hydrolysis treatment
Total alkaline protease	<0.001	0.0381	0.8467	0.2237
Trypsin	<0.001	0.419	0.3013	0.1461
Chymotrypsin	<0.001	<0.001	0.6056	0.0484
Leucine aminopeptidase	<0.001	0.0169	0.2618	0.7807
Alkaline phosphatase	<0.001	0.0952	0.5947	0.1142

Data are mean ± SD (n=9). Factors are: i) Age of fish larvae; ii) Microalgae (with or without microalgae inclusion), iii) Inclusion level (microalgae dietary inclusion level), and iv) Hydrolysis treatment (crude biomass vs hydrolysed biomass).

Table 8. Microvillar morphology of the anterior intestine of gilthead seabream fry fed with the experimental diets for 40 days.

	ML (μm)	MD (μm)	EA (μm²)	TAS (μm²)
CT	1.84 ± 0.16 a	0.12 ± 0.01 a	27.04 ± 3.21 b	1012.01 ± 157.51 a
C-5	2.06 ± 0.13 b	0.14 ± 0.01 c	22.56 ± 3.70 a	1089.77 ± 194.24 a
C-10	1.88 ± 0.19 a	0.14 ± 0.01 c	22.65 ± 3.64 a	1021.84 ± 148.08 a
H-5	2.09 ± 0.13 b	0.15 ± 0.01 d	22.92 ± 3.39 a	1079.58 ± 158.57 a
H-10	2.89 ± 0.43 c	0.13 ± 0.01 b	29.84 ± 2.94 c	1930.06 ± 311.07 b
P-value	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. ML: microvilli length; MD: microvilli diameter; EA: enterocyte apical area; TAS: enterocyte absorption surface. Values are mean ± SD of 60 measures obtained from three specimens per replicate tank. Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

Table 9. Summary of the results in the discriminant analysis (DA) of the different dietary treatments considering digestive enzyme activities and ultrastructural analysis of the intestinal mucosa as predictor variables.

Discriminant function	Eigenvalue	Relative percentage	Canonical correlation	Wilk´s λ	χ^2	Degree of freedom	*P*-value
1	8.31057	81.45	0.94477	0.0358087	760.8052	16	<0.001
2	1.83474	17.98	0.80451	0.3334	250.9873	9	<0.001

Table 10. Antimicrobial activities in liver extracts of gilthead seabream fry fed with the experimental diets for 40 days.

	Antiprotease (% g tissue^{-1})	Protease (% µg tissue^{-1})	Peroxidase (U µg tissue^{-1})	Lysozyme (U µg tissue^{-1})	Bactericidal activity (% µg tissue^{-1})
CT	0.09 ± 0.04	0.02 ± 0.01	0.68 ± 0.06	0.05 ± 0.01 a	0.32 ± 0.12
C-5	0.09 ± 0.04	0.46 ± 0.24	0.86 ± 0.10	0.06 ± 0.01 a	0.48 ± 0.16
C-10	0.07 ± 0.02	0.03 ± 0.01	0.74 ± 0.07	0.05 ± 0.01 a	0.42 ± 0.09
H-5	0.08 ± 0.01	0.01 ± 0.00	0.67 ± 0.07	0.04 ± 0.00 a	0.37 ± 0.07
H-10	0.07 ± 0.03	0.34 ± 0.18	0.84 ± 0.11	0.16 ± 0.06 b	0.72 ± 0.21
P-value	*0.9851*	*0.1979*	*0.4330*	*0.0194*	*0.1564*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of 9 measures obtained from three specimens per replicate tank. Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

Captions to Figures

Figure 1. The final body weight of gilthead seabream fry fed with experimental diets for 40 days. Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

Figure 2. Muscle thiobarbituric acid-reactive substances (TBARS) content of gilthead seabream fry after 40-day feeding trial (mean ± standard deviation, n = 12). Values with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$). CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

Figure 3. Enzyme activities measured in intestinal extracts of *S. aurata* fry fed with the experimental diets for 40 days. Values are mean ± SD (n=9, nine pools of intestines per dietary treatment). Values in the same day with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$). CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

Figure 4. Comparative SEM (A) and TEM (B) micrographs from the anterior intestinal regions of *S. aurata* fry at the end of the feeding trial. CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. (SEM bar: 30 μm; TEM bar: 2 μm).

Figure 5. Plot of the first two discriminant functions established by the discriminant analysis (DA) of digestive functionality analysis of *S. aurata* fry fed with the experimental diets.

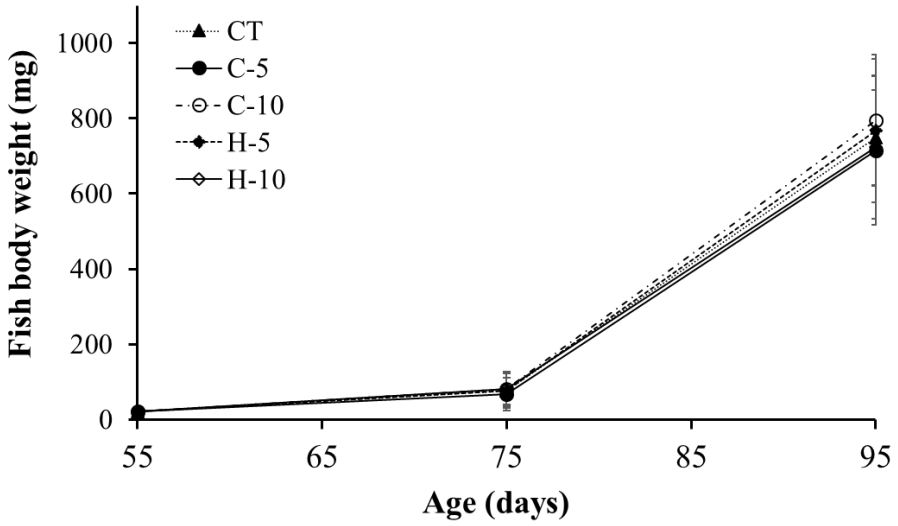
CT
C-5
C-10
H-5
H-10
Fish body weight (mg)
1000
800
600
400
200
0
55
65
75
85
95
Age (days)

Fig. 1

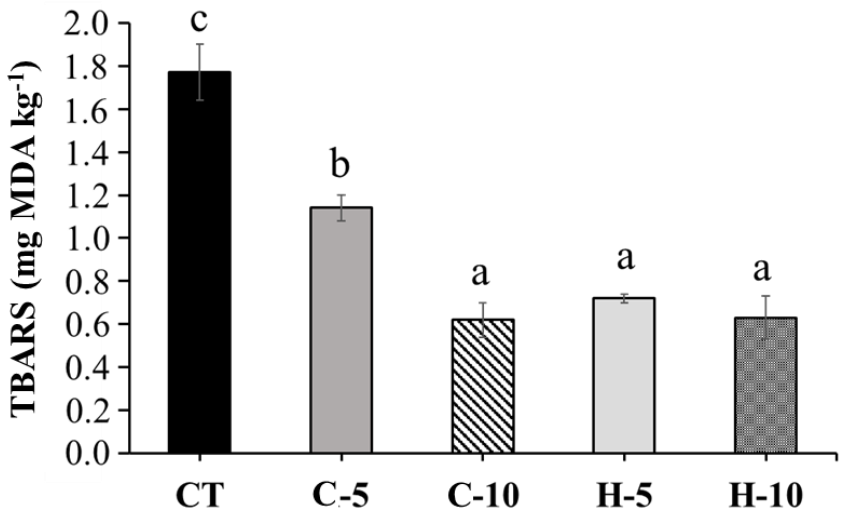
TBARS (mg MDA kg-1)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
c
b
a
a
a
CT
C-5
C-10
H-5
H-10

Fig. 2

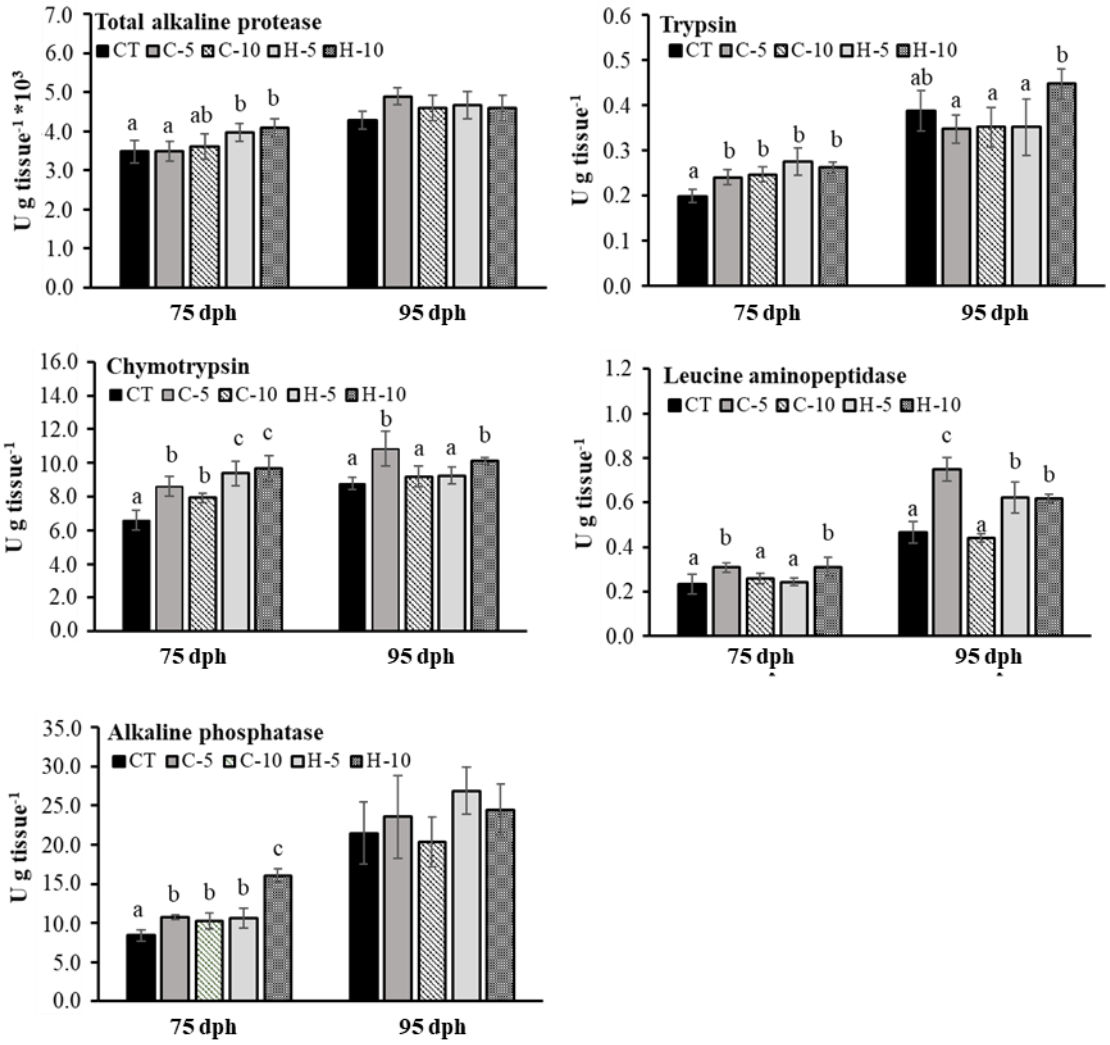
Total alkaline protease
CT C-5 C-10 H-5 H-10
U g tissue-1 *10³
75 dph
95 dph
Trypsin
U g tissue-1
Chymotrypsin
Leucine aminopeptidase
Alkaline phosphatase

Fig. 3

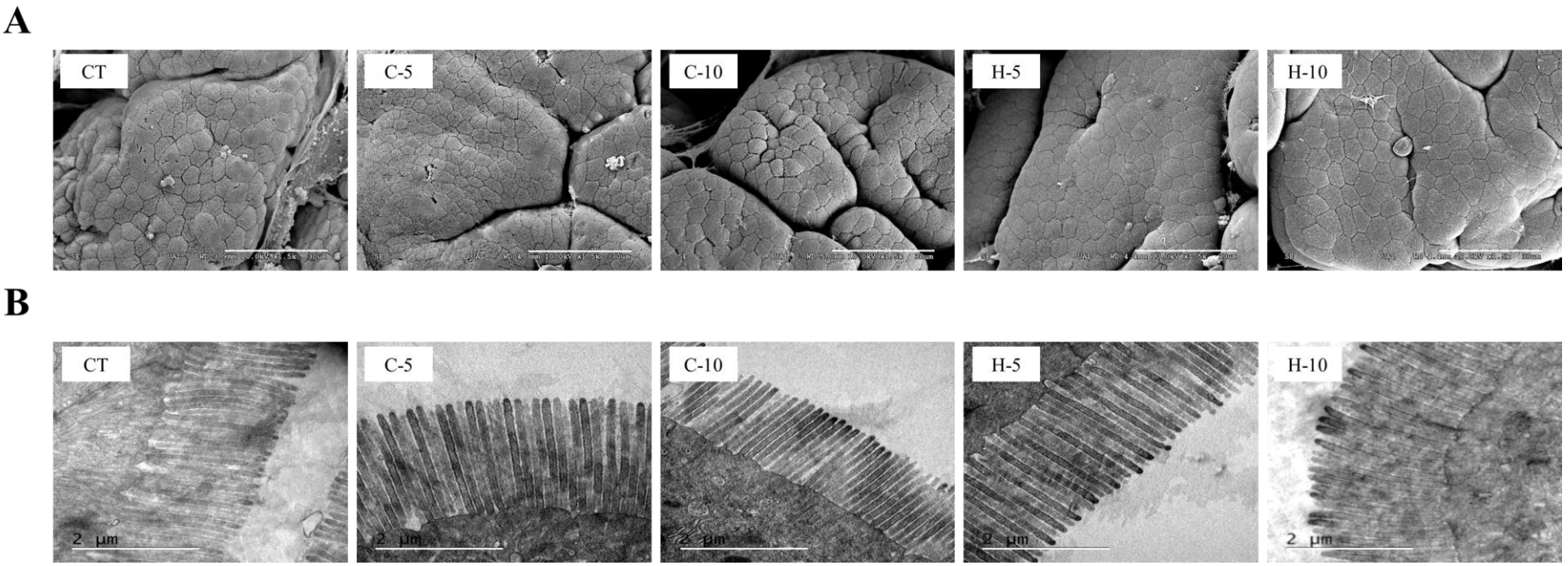

Fig. 4

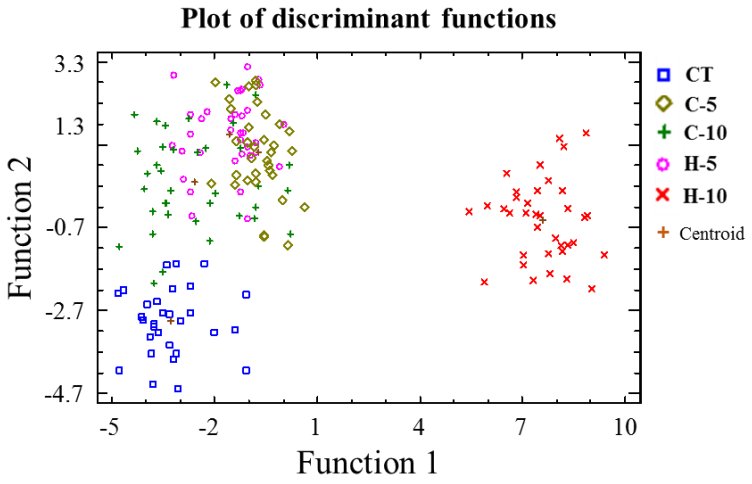
Plot of discriminant functions
3.3
1.3
-0.7
-2.7
-4.7
Function 2
-5
-2
1
4
7
10
Function 1
CT
C-5
C-10
H-5
H-10
Centroid

Fig. 5