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64 Abstract

The aim of this work was to evaluate the effects of the dietary inclusion of *Arthrospira* sp. enzyme hydrolysate on gilthead seabream (*Sparus aurata)* juveniles in a 128-day feeding trial. Algal hydrolysate was tested at low inclusion level, namely, 2 and 4%, against a control diet without *Arthrospira* sp. supplementation. At the end of the feeding trial, fish body weight was recorded for growth evaluation. No significant differences were found among the experimental groups regarding growth performance or nutrient utilization, despite the fact that those animals fed with diets enriched with *Arthrospira* displayed higher trypsin, chymotrypsin, and leucine aminopeptidase enzyme activities, compared to fish fed with control diet. The ultrastructural study of the intestinal mucosa also revealed increased microvilli length and absorptive capacity in fish fed with *Arthrospira* sp. diets, especially at 4% inclusion level. *Arthrospira* supplementation was also responsible for lower lipid oxidation in muscle tissue, as well as for remarkable colour differences in skin, compared to control animals.
These results suggest, overall, that low dietary supplementation with this microalgal hydrolysate might improve not only the intestinal ultrastructure and functionality but also muscle pigmentation and antioxidant capacity of juvenile gilthead seabream.

65 Keywords separated by ' - '

Growth performance - Intestine ultrastructure - Functional additive - Microalgae hydrolysate

66 Foot note information

• *Sparus aurata* was fed with *Arthrospira* hydrolysate at low inclusion level (2 and 4%) for 128 days.
• *Arthrospira* sp. enzyme hydrolysate enhanced intestinal functionality in juvenile gilthead seabream.
• Algal enzyme hydrolysate in diets modified skin pigmentation and prevented muscle lipid oxidation.

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Evaluation of *Arthrospira sp.* enzyme hydrolysate as dietary additive in gilthead seabream (*Sparus aurata*) juveniles

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Abstract

The aim of this work was to evaluate the effects of the dietary inclusion of *Arthrospira* sp. enzyme hydrolysate on gilthead seabream (*Sparus aurata)* juveniles in a 128-day feeding trial. Algal hydrolysate was tested at low inclusion level, namely, 2 and 4%, against a control diet without *Arthrospira* sp. supplementation. At the end of the feeding trial, fish body weight was recorded for growth evaluation. No significant differences were found among the experimental groups regarding growth performance or nutrient utilization, despite the fact that those animals fed with diets enriched with *Arthrospira* displayed higher trypsin, chymotrypsin, and leucine aminopeptidase enzyme activities, compared to fish fed with control diet. The ultrastructural study of the intestinal mucosa also revealed increased microvilli length and absorptive capacity in fish fed with *Arthrospira* sp. diets, especially at 4% inclusion level. *Arthrospira* supplementation was also responsible for lower lipid oxidation in muscle tissue, as well as for remarkable colour differences in skin, compared to control animals.

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Keywords Growth performance · Intestine ultrastructure · Functional additive · Microalgae hydrolysate

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Introduction

The interest in microalgae has increased strongly in the last years, given that they have valuable potential for reducing the dependence on unsustainable conventional raw ingredients in aquafeeds (Shah et al. 2018). The use of microalgae in aquaculture can be approached from two perspectives: on one hand, taking into account their nutritive value as protein and lipid sources, and on the other, considering that microalgae also have plenty of substances with potential bioactive effects. Abundant literature on the first consideration is available, but it is likely that the main constraint for extensive utilization of microalgae consists of the fact that any satisfactory alternative feed ingredient must be able to supply comparable nutritional value at competitive cost. To date, this is far from being achieved, given that any large-scale practical utilization of microalgae relies on a significant reduction in production costs. With regard to the second aspect mentioned above, growing interest is currently being paid to the fact that microalgae can accumulate useful metabolites, normally at relatively low concentration, with potentially bioactive effects. Thereby, the interest in microalgae as potential nutraceutical additive in aquafeeds is increasing considerably (Chakraborty and Hancz 2011; Cardinaletti et al. 2018; Shah et al. 2018).

Numerous studies have reported that microalgae can be used as dietary ingredient or additive in aquafeeds without exerting negative impacts on fish growth and nutrient utilization (De Cruz et al. 2018; Perez-Velazquez et al. 2018) and even yielding valuable effects on lipid metabolism (Robin and

Highlights

- *Sparus aurata* was fed with *Arthrospira* hydrolysate at low inclusion level (2 and 4%) for 128 days.
- *Arthrospira* sp. enzyme hydrolysate enhanced intestinal functionality in juvenile gilthead seabream.
- Algal enzyme hydrolysate in diets modified skin pigmentation and prevented muscle lipid oxidation.

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Vincent 2003), fish gut functionality (Vizcaíno et al. 2016, 2018), and oxidative status and lipid utilization in different fish species (Kiron 2012; Teimouri et al. 2013; Roy and Pal 2015; Amer 2016). In addition, positive effects have been reported in rainbow trout (*Oncorhynchus mykiss*) related to pigmentation attributes (Teimouri et al. 2013) and lipid peroxidation (Teimouri et al. 2016).

The genus *Arthrospira* (filamentous *Cyanobacteria*) is known for its high protein content, up to 70% on dry matter basis (Santigosa et al. 2011; Macias-Sancho et al. 2014; Ansarifard et al. 2018), with amino acid profiles comparable to those found in some reference feed proteins (Becker 2007). *Arthrospira* is also rich in polyunsaturated fatty acids (PUFAs), mainly gamma-linolenic acid (18:3n6) (Ronda et al. 2012), as well as in vitamins (A and B_{12}), minerals, and pigments with acknowledged antioxidant activity, such as carotenoids (Pugh et al. 2001; El-Sheekh et al. 2014; Adel et al. 2016; Velasquez et al. 2016), phycobilins, and phycocyanins (Mahmoud et al. 2018; Takyar et al. 2019). Thus, the dietary inclusion of *Arthrospira* has been evaluated in different fish species (Hussein et al. 2013; Kim et al. 2013; Teimouri et al. 2013; Velasquez et al. 2016; De Cruz et al. 2018; Perez-Velazquez et al. 2018), these studies reporting, overall, a lack of negative effects on growth performance or nutrient utilization but even favourable impacts on fish physiology. However, the potential effects of *Arthrospira* sp. on fish growth and objective quality parameters of Mediterranean fish species remain virtually unknown.

However, microalgae also display certain disadvantages from a nutritional point of view, such as the structure and composition of their cell wall, which is a protective barrier that reduces the bioavailability of the intracellular nutrients (Wu et al. 2017). The efficiency of marine animals to digest the cell walls depends on the carbohydrate composition, on how they are linked to each other, and on the existence of suitable digestive enzymes. Overall, herbivorous and omnivorous species possess a wide range of carbohydrases, but carnivorous fish do not, and this fact should be taken into consideration when it comes to formulating aquafeeds. Consequently, it may be reasonable to think that any strategy aimed at improving the bioavailability of the inner compounds, not only of *Arthrospira* sp. but of any other species, might enable to include microalgae at low inclusion level in aquafeeds. Several procedures have been evaluated with the aim of releasing inner components of microalgae (Tibbetts et al. 2017; Agboola et al. 2019; Teuling et al. 2019), but when it comes to large-scale cell lysis, enzymatic hydrolysis is one of the most promising strategies, not least owing to its economic viability. By following this procedure, even low inclusion level of enzyme-hydrolysed microalgae in aquafeeds might well improve the physiological aspects in fish in a manner similar to including higher amounts of raw microalgae (Tchorbanov and Bozhkova 1988). To our knowledge, despite the potential of this procedure to increase nutrient bioavailability and functional properties, the use of microalgae enzymatic hydrolysates in aquafeeds remains unexplored. Thus, the production of microalgal hydrolysates is a promising strategy that deserves further research efforts.

Protein hydrolysates are believed to be more effective than intact protein or free amino acids from a nutritional point of view. The enzymatic hydrolysis of proteins results in the formation of a mixture of free amino acids, di-, tri-, and oligopeptides, and enhances the occurrence of polar groups and the solubility of hydrolysate compounds. The dietary use of protein hydrolysates of different origins in some species of farmed fish has proved several positive bioactive effects, such as antioxidant, antimicrobial, or anti-inflammatory, and beneficial effects on the functionality of the intestinal mucosa (Leduc et al. 2018; Zamora-Sillero et al. 2018). In the case of algae protein, enzymatic hydrolysis could release low molecular weight bioactive peptides and free amino acids, which might enable not only increased bioavailability but also lead to potential positive physiological effects (Morris et al. 2007; Chalamaiah et al. 2012; Montone et al. 2018; Wang et al. 2018).

In this piece of research, we hypothesise that *Arthrospira* sp. enzyme hydrolysate might improve some parameters related to growth performance, muscle lipid oxidation, skin pigmentation, and digestive functionality of juvenile gilthead seabream when added a low dietary inclusion level. The overall objective of this study is focused specifically on the assessment of the potential effects of low level of microalgae protein hydrolysate as functional additive in practical diets for juvenile fish of this Mediterranean species.

Materials and methods

Arthrospira sp. hydrolysate

Arthrospira sp. hydrolysate was produced starting from a sludge containing up to 150 g L^{-1} of microalgae biomass after performing an enzymatic hydrolysis with a mixture of commercial proteases under controlled conditions (pH 8.0 and 50 °C under continuous stirring) for 4 h providing 0.2% w/w proteases (Alcalase 2.4 L and Flavourzyme 1000 L from Novozymes A/S, Denmark), following a modification of the method described by Saadaoui et al. (2019). Alcalase 2.4 L is a microbial protease of *Bacillus licheniformis* with endopeptidase activity. A main component of the commercial preparation is the serine protease subtilisin A. The specific activity of Alcalase 2.4 L is 2.4 Anson Unit (AU) per gramme. One AU is the amount of enzyme, which, under standard conditions, digests haemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with the Folin reagent as one mequivalent of tyrosine Q1

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157 released per minute. Flavourzyme 1000 L is a protease com-
158 plex of *Aspergillus oryzae* that contains both endo- and
159 exoprotease activities. It has an activity of 1.0 leucine amino-
160 peptidase (LAPU) unit g^{-1}. One LAPU is the amount of en-
161 zyme that hydrolyses 1 μmol of leucine-p-nitroanilide per
162 minute. Immediately after the hydrolysis, the reaction mixture
163 was heated at 80 °C for 15 min in order to inactivate the
164 proteolytic enzymes. Total free amino acids were quantified
165 spectrophotometrically at 340 nm using L-leucine as standard
166 (Church et al. 1983). In brief, triplicate samples of 50 μL were
167 withdrawn from microalgal protein hydrolysate, and 50 μL of
168 20% trichloroacetic acid (TCA) were added with the purpose
169 of stopping the enzyme reaction. Afterwards, protein precipi-
170 tates were discarded by centrifugation (12,000 rpm, 15 min at
171 4 °C), and the supernatants were stored at − 20 °C until further
172 analysis. Finally, SDS-PAGE (Laemmli 1970) for crude
173 *Arthrospira* sp. meal and its protein hydrolysate was per-
174 formed in order to identify the protein fractions and their mo-
175 lecular weight.

176 **Experimental diets**

177 Three isonitrogenous (450 g crude protein kg^{-1}) and isolipidic
178 (170 g crude lipid kg^{-1}) experimental feeds were formulated,
179 control without microalgae (CT), plus AH-2 and AH-4, contain-
180 ing 2% and 4% (DM basis) *Arthrospira* sp. hydrolysate, respec-
181 tively. The formulation and chemical composition of the exper-
182 imental diets are shown in Table 1. Before adding fish oil and
183 diluted choline chloride, feed ingredients were finely ground and
184 mixed in a vertical helix mixer (Sammic 13 M-11, 5-L capacity,
185 Sammic SA, Spain) for 20 min. Then the algae hydrolysate was
186 added, and water content was adjusted to provide 400 mL per kg
187 of ingredient mixture to obtain a homogenous dough. The dough
188 was passed through a single screw laboratory extruder (Miltenz
189 51SP, JS Conwell Ltd., New Zealand) in order to obtain 2- and 3-
190 mm pellets. The feeds were dried in a 12-m^3 drying chamber
191 with forced-air circulation (Airfrio, Spain) at 30 °C for 24 h and
192 stored at − 20 °C until use. An attractant premix was added
193 (50 g kg^{-1}) to improve feed palatability (according to Barroso
194 et al. 2013). The experimental diets were produced by
195 LifeBioencapsulation SL (Spin-off, Universidad de Almería,
196 Spain).

197 **Feeding trial and sampling**

198 Feeding trial was carried out at the *Servicios Centrales de*
199 *Investigación en Cultivos Marinos* (SCI-CM, CASEM,
200 Universidad de Cádiz, Puerto Real, Spain). All experimental
201 procedures complied with the Guidelines of the European
202 Union (Directive 2010/63/UE) regarding the use of laboratory
203 animals. The competent Ethical Committee approved the ex-
204 perimental procedures involving the use of fish (Junta de
205 Andalucía, reference number 06/02/2020/011). A total of
206 180 gilthead seabream juveniles (20 g average body weight)
207 were selected and randomly distributed in 9 tanks (triplicate
208 tanks per dietary treatment) of 75-L capacity (400 g average
209 biomass per tank). Fish were fed with a commercial diet
210 (Skretting España, 45% crude protein, 19% crude lipid)

Table 1 Ingredient composition and proximate composition (g kg^{-1} on dry matter basis) of the experimental diets t1.1

	CT	AH-2	AH-4	
				t1.2
Ingredients				t1.3
Fishmeal[1]	374.2	358.4	340.6	t1.4
Arthrospira sp. hydrolysate (g dry matter)[2]		20	40	t1.5
Attractant premix[3]	50	50	50	t1.6
Wheat gluten[4]	95	95	95	t1.7
Soybean meal[5]	165	165	165	t1.8
Fish oil	72.8	73.9	74,9	t1.9
Soybean oil	28	28	28	t1.10
Wheat flour[6]	170	164.7	161.5	t1.11
Betaine	5	5	5	t1.12
Vitamins and minerals premix[7]	20	20	20	t1.13
Binder (guar gum)[8]	20	20	20	t1.14
Proximate composition				t1.15
Crude protein	449.9	450.3	449.5	t1.16
Crude lipid	169.7	170.1	170.3	t1.17
Ash	70.8	69.2	67.2	t1.18
Crude fibre	34.7	34.6	34.5	t1.19
NfE[9]	274.8	275.9	278.5	t1.20

CT control diet, *AH-2* 2% *Arthrospira* hydrolysate-supplemented diet, *AH-4* 4% *Arthrospira* hydrolysate-supplemented diet

[1] Protein, 69.4%; lipid, 12.3%; Norsildemel (Bergen, Norway)

[2] Liquid product containing 150 g microalgae meal L^{-1}

[3] 50% squid meal, 25% shrimp meal, 25% krill meal

[4] Protein, 76.0%; lipid, 1.9%

[5] Protein, 50.0%; lipid, 1.0%

[6] Protein, 12.0%; lipid, 2.0%

[7] Vitamin and mineral premix: vitamins (IU or mg kg^{-1} premix): vitamin A (retinyl acetate), 2000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E, 10,000 mg; vitamin K_3 (menadione sodium bisulphite), 2500 mg; vitamin B1(thiamine hydrochloride), 3000 mg; vitamin B_2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 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 premix)

[8] EPSA (Sevilla, Spain)

[9] *NfE* nitrogen-free extract calculated as 100 − (% crude protein + % ether extract + % ash + % crude fibre)

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211 during a 15-day acclimation period. Afterwards, experimental 212 diets were offered twice per day (9:00 and 17:00) at 2% of the 213 biomass, until triplication of the initial body weight. The 128- 214 day feeding trial was carried out in a flow-through filtered 215 (1 μm) seawater system sterilized by UV, under constant tem- 216 perature (19.0 ± 1.1 °C), salinity (35 ± 1 ‰), and natural pho- 217 toperiod (light:dark, LD; from 11:13 h in February to 13:11 h 218 in May). The water in all the tanks was oxygen-saturated (> 219 90% O_2 saturation) with air stones. Water ammonia (< 220 0.1 mg L^{-1}), nitrite (< 0.2 mg L^{-1}), and nitrate (< 221 50 mg L^{-1}) were determined with commercial kits (SERA 222 GmbH, Heinsberg, Germany).

223 Fish were individually weighed every 2 weeks after a 24-h 224 fasting period in order to determine growth and feed utiliza- 225 tion parameters. At the end of the feeding trial, 16 fish per tank 226 were killed by anaesthetic overdose (200 mg L^{-1} clove oil) 227 followed by spine severing. Sampled fish were dissected, and 228 the digestive tract and dorsal muscle were removed. Dorsal 229 muscle samples were carefully washed, dried, and packaged 230 in transparent sterile polyethylene bags and stored in a cold 231 room (4 ± 1 °C). Muscle samples were withdrawn from each 232 dietary treatment at day 1 and 8 of cold storage. Colour pa- 233 rameters were determined on the right side of the anterior 234 dorsal skin of fish, and then a portion of muscle tissue (5 g) 235 was used for lipid oxidation determination (TBARS). The rest 236 of individual muscle samples were freeze-dried and stored at 237 − 20 °C for further analysis of muscle proximate composition. 238 For digestive enzyme activity determinations, intestines from 239 nine fish per tank were randomly pooled to obtain three enzy- 240 matic extracts from each experimental tank. Intestine samples 241 were homogenized in distilled water at 4 °C (0.5 g mL^{-1}). 242 Supernatants were obtained after centrifugation (11,200 *xg*, 243 12 min, 4 °C) and stored in aliquots at − 20 °C until further 244 use. Total soluble protein was determined using bovine serum 245 albumin as standard (according to Bradford 1976). Finally, the 246 intestines of three specimens from each tank were collected 247 for examination by transmission (TEM) and scanning (SEM) 248 electron microscopy.

249 Growth performance, nutrient utilization, 250 and somatic indices

251 Growth performance was assessed by different parame- 252 ters according to the following formulae: daily gain (DG, 253 g day^{-1}) = (Wf − Wi) / days; specific growth rate (SGR, 254 %) = (Ln Wf − Ln Wi) / days × 100; condition factor 255 (K) = (Wf / SL^3) × 100, where *Wf* was the final weight 256 (g), *Wi* was the initial weight (g), and *SL* was the stan- 257 dard length.

258 Nutrient utilization indices were estimated as follows: feed 259 conversion ratio (FCR) = total feed intake on dry basis (g) / 260 weight gain (g) and protein efficiency ratio (PER) = WG / total 261 protein ingested (g), where *WG* was the weight gain (g).

Proximate analysis 262

Proximate analysis of feeds and muscle samples were carried 263 according to AOAC (2000) for dry matter and ash, whereas 264 crude protein (N × 6.25) was determined by using elemental 265 analysis (C:H:N) (Fisons EA 1108 analyzer, Fisons 266 Instruments, USA). Total lipid content was analysed follow- 267 ing the procedure described by Folch (1957). 268

Skin colour determinations 269

Instrumental colour was measured in triplicate on the right 270 side of the dorsal fish skin by L*, a*, and b* system (CIE 271 1978), using a Minolta chroma meter CR-400 (Minolta, 272 Japan). The lightness (L*, on a 0–100 point scale from black 273 to white), redness (a*, estimates the position between red, 274 positive values, and green, negative values), and yellowness 275 (b*, estimates the position between yellow, positive values, 276 and blue, negative values) were recorded. 277

Muscle lipid oxidation 278

Lipid oxidation in muscle samples was estimated by thiobar- 279 bituric acid-reactive substances (TBARS) at 1 and 8 dpm 280 (days post-mortem). TBARS were measured in muscle sam- 281 ples according to the method of Buege and Aust (1978). 282 Samples (1 g) were homogenized in 4 mL 50 mM 283 NaH_2PO4, 0.1% (v/v) Triton X-100 solution. The mixture 284 was centrifuged (10,000 *xg*, 20 min, 4 °C). Supernatants were 285 mixed in a 1:5 ratio (v/v) with 2-tiobarbituric acid (TBA) 286 reagent (0.375% w/*v* TBA, 15% w/v TCA, 0.01% w/v 2,6- 287 di-tert-butyl-4-methylphenol (BHT) and 0.25 N HCl). The 288 mixture was heated for 15 min and centrifuged (3600 *xg*, 289 10 min, 4 °C), and the absorbance of supernatants was mea- 290 sured at 535 nm. The amount of TBARS was expressed as mg 291 of malondialdehyde (MDA) per kg of muscle after comparing 292 with a MDA standard. 293

Digestive enzyme activities 294

Total alkaline protease activity in the digestive extracts was 295 spectrophotometrically determined using 5 g L^{-1} casein in 296 50 mM Tris-HCl (pH 9.0) as substrate, according to Alarcón 297 et al. (1998). One unit of total protease activity was defined as 298 the amount of enzyme that released 1 μg of tyrosine per min- 299 ute in the reaction mixture, considering an extinction coeffi- 300 cient for tyrosine of 0.008 μg^{-1} mL^{-1} cm^{-1}, measured at 301 280 nm. Trypsin and chymotrypsin activities were determined 302 by using 0.5 mM BAPNA (N-a-benzoyl-DL-arginine-4-p- 303 nitroanilide) as substrate, according to Erlanger et al. (1961), 304 and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-p- 305 nitroanilide) according to DelMar et al. (1979); both substrates 306 dissolved in 50 mM Tris-HCl and 10 mM $CaCl_2$ buffer 307

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(pH 8.5). Leucine aminopeptidase activity was quantified according to Pfleiderer (1970), using 2 mM L-leucine-p-nitroanilide (LpNa) in 100 mM Tris-HCl buffer, pH 8.8, as substrate. For trypsin, chymotrypsin, and leucine aminopeptidase activities, one unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 μmol of p-nitroanilide (pNA) per minute, measured spectrophotometrically at 405 nm, considering an extinction coefficient of 8800 M cm^{-1}. Alkaline phosphatase activity was determined by using p-nitrophenyl phosphate in 1 M diethanolamine, 1 mM $MgCl_2$ buffer, pH 9.5, as substrate, following the method described in Bergmeyer (1974). For alkaline phosphatase, one unit of enzyme activity was defined as the amount of enzyme that releases 1 μg of nitrophenyl per min, considering a molar extinction coefficient of 17,800 M cm^{-1} for p-nitrophenol measured at 405 nm.

Ultrastructural study of intestinal mucosa

Intestine samples were collected for electron microscopy evaluation. Samples for scanning electron microscopy (SEM) were previously washed with 1% S-carboxymethyl-L-cysteine (Sigma Chem.) for 20 s, with the aim of removing the epithelial mucus. Afterwards, the samples were fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.2) for 24 h, after which samples were washed and dehydrated in graded ethanol. Then samples were critical point dried with absolute ethanol as intermediate fluid and CO_2 as transition fluid (CDP 030 Critical point dryer, Leica Microsystems, Spain). After drying, specimens were mounted on supports and fixed with graphite (PELCO Colloidal Graphite, Ted Pella INC., USA) and then 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). Samples for transmission electron microscopy (TEM) were fixed (4 h, 4 °C) in 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5. Next, intestine sections were washed with PBS for 20 min, and then, a post-fixation step with 20 g L^{-1} osmium tetroxide was carried out. Then, samples were dehydrated by consecutive immersion (20 min each) in ethanol solution of gradients ranging from 50 to 100% (v/v). Next, samples were embedded for 2 h, in 1:1 mixture of Epon resin and 100% (v/v) ethanol under continuous shaking, and then they were included in pure Epon resin and polymerized at 60 °C. Finally, the ultrathin sections were placed on a 700 Å copper mesh and stained with uranyl acetate and lead citrate. The mesh observation was performed with a Zeiss 10C TEM at 100 Kv (Carl Zeiss, Spain). Visualization fields were recorded at ×16,000 magnification. SEM and TEM visualization fields were recorded, and digital images were analysed using UTHSCSA ImageTool software (University of Texas Health Science Center, San Antonio, TX, USA). Microvilli length (ML) and microvilli diameter (MD) and the number of microvilli within 1-μm 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).

Statistical analysis

All assays were repeated at least three times with three replicates. Data were expressed as mean ± SE. Comparison of means was carried out by one-way ANOVA with a 5% level of probability ($p < 0.05$) followed by a multiple comparison test. Data in percentage (%) were arcsine (×1/2)-transformed, checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). When the data did not meet the ANOVA assumptions, a Kruskal-Wallis one-way analysis of variance on ranks was used. When the Kruskal-Wallis test showed significance, and Dunn's method of multiple comparisons was used to compare individual medians. All statistical analyses were performed with Statgraphics Plus 4.0 (USA) software.

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Results

Characterization of the protein hydrolysate of *Arthrospira* sp.

Figure 1 shows the proteinograms of raw *Arthrospira* sp. meal and its protein hydrolysate. Raw meal showed a complex protein profile made up of several fractions with a wide range from 13 to 86 kDa. However, microalgal protein hydrolysate shows only two protein fractions (57 and 39 kDa) in the range of molecular weight visualized in the proteinogram. In addition, quantification of total free amino acids revealed that *Arthrospira* sp. hydrolysate contained higher level of free amino acids (84.06 ± 3.23 mg leucine equivalents $(100\ mg)^{-1}$ protein) than those found in the raw microalgae biomass (31.5 ± 3.09 mg leucine equivalents $(100\ mg)^{-1}$ protein).

Growth performance and nutrient utilization

Growth of gilthead seabream juveniles fed experimental diets for 128 days is shown in Fig. 2.

All dietary groups showed similar final body weight, DG, and SGR, without significant differences ($p > 0.05$), although mean values were slightly lower in AH-2 and AH-4 groups. Similarly, no significant differences were observed in FCR and PER mean values (Table 2).

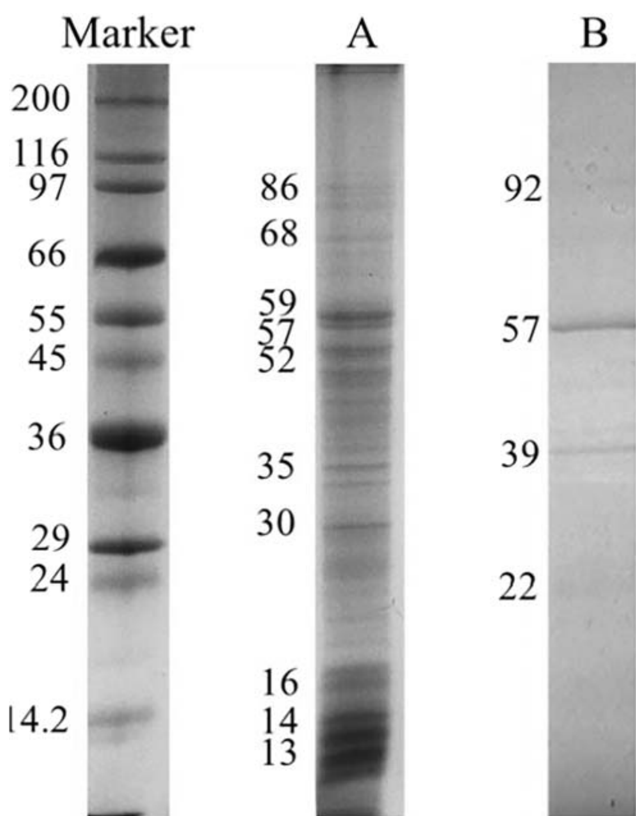

Fig. 1 SDS-PAGE of the raw *Arthrospira* sp. biomass (**a**) and its protein hydrolysate (**b**). Figures at the left of each proteinogram indicate the molecular mass (kDa) of the main protein fractions separated. Marker: 5 μL of wide range molecular weight marker (S-84445, Sigma, St. Louis, USA) ranging from 6.5 (aprotinin, bovine lung) to 200 kDa (myosin, porcine heart)

Muscle proximate composition 402

Muscle chemical composition is shown in Table 3. Protein content increased significantly in fish fed with diets supplemented with *Arthrospira* hydrolysate, especially in AH-2 group, whereas a significant decrease in the lipid content was observed in fish fed with AH-2 and AH-4 diets. Moisture and ash content were similar among dietary treatments. 403–409

Instrumental skin colour determinations 410

Initial L* values were significantly higher in AH-2-fed fish, compared to CT and AH-4 groups (Table 4). After 8 days of cold storage, values remained stable in AH-2 and AH-4, whereas in CT significantly decreased ($p < 0.001$). CT presented a* negative values indicating a skin greenish coloration. However, values for AH-2 and AH-4 were positive which evidenced a slightly red coloration, though they decreased significantly at 8 dpm ($p = 0.040$ and < 0.001). Skin b* values in CT were positive and sharply decreased during storage under refrigeration. Nevertheless, values of AH-2 and AH-4 significantly increased, indicating a yellowish colour of the skin. 411–422

Muscle lipid oxidation (TBARS) 423

Muscle TBARS content in CT group showed significantly higher values (Table 4). Muscle lipid oxidation increased during cold storage ($p = 0.015$ and $p = 0.019$), although TBARS values were significantly lower at any sampling time in specimens fed with *Arthrospira* hydrolysate compared to CT fish ($p = 0.019$ and $p = 0.015$). 424–429

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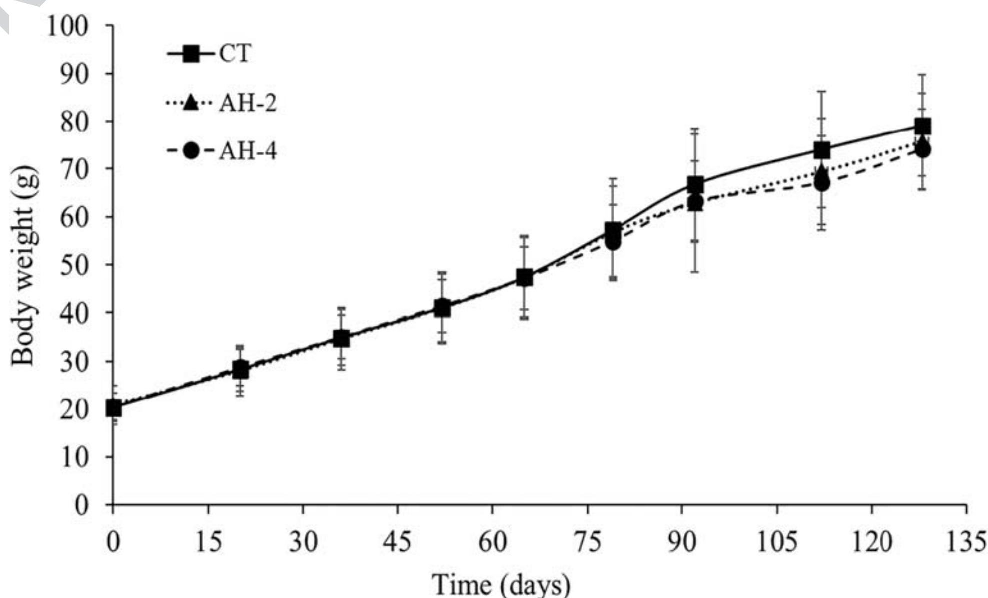

Fig. 2 Time course of changes in body weight of fish fed with the experimental diets. *CT* control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate

t2.1 t2.2 t2.3 t2.4 **Table 2** Growth performance and nutrient utilization parameters of gilthead seabream juveniles fed with the experimental diets during the 128-d feeding trial

	CT			AH-2			AH-4			*p*
Growth and nutrient utilization										
Initial body weight (g)	20.32	±	0.48	20.80	±	0.52	20.51	±	0.36	*0.7701*
Final body weight (g)	79.09	±	1.75	75.80	±	1.38	74.06	±	1.15	*0.0529*
Fulton's condition factor	1.80	±	0.03	1.81	±	0.05	1.77	±	0.04	*0.9404*
Daily gain (DG, g day^{-1})	0.46	±	0.06	0.43	±	0.01	0.42	±	0.02	*0.5803*
Specific Growth Rate (SGR)	1.05	±	0.13	0.99	±	0.04	0.98	±	0.05	*0.8948*
Feed Conversion Ratio (FCR)	0.47	±	0.03	0.41	±	0.04	0.42	±	0.05	*0.5789*
Protein efficiency ratio (PER)	2.15	±	0.15	2.49	±	0.24	2.45	±	0.33	*0.6054*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate tanks

430 **Digestive enzyme activities**

431 Trypsin, chymotrypsin, and leucine aminopeptidase activities 432 significantly increased in fish fed with *Arthrospira* hydrolysate- 433 supplemented diets ($p = 0.001$, $p = 0.001$, and $p < 0.001$, respec- 434 tively) compared to control fish (Table 5). Fish fed with AH-4 435 showed the highest enzyme activity levels. Total alkaline prote- 436 ase and alkaline phosphatase activities did not differ among ex- 437 perimental groups ($p = 0.160$ and $p = 0.844$).

438 **Ultrastructural study of the intestinal mucosa**

439 TEM and SEM observations evidenced that all specimens 440 presented intestinal mucosa without any evidence of abnor- 441 mality (Fig. 3). Nevertheless, the morphometric analysis of the 442 intestinal microvilli carried out on both TEM and SEM images 443 evidenced a significant increase in microvilli length (ML) and 444 microvilli diameter (MD) in fish fed with AH-4 diet. 445 Enterocyte apical area values were similar in all dietary treat- 446 ments ($p = 0.211$), but total enterocyte absorption surface 447 (TAS) was significantly higher in fish fed with AH-4 diet 448 compared to CT group ($p < 0.001$) (Table 6).

449 ## Discussion

450 The use of *Arthrospira* hydrolysates in aquafeeds arise as a 451 novel strategy aimed at increasing the nutritional and 452 functional properties of the original raw biomass, by turning 453 the proteins into low molecular weight peptides and free ami- 454 no acids with higher bioavailability (Chalamaiah et al. 2012). 455 The existence of low molecular mass molecules and free ami- 456 no acids as a result of the enzymatic hydrolysis has been 457 proposed as an interesting dietary supplement for 458 aquacultured fish (Xu et al. 2016). Indeed, the potential ben- 459 eficial effects derived from the use of protein hydrolysates in 460 aquafeeds have been proven previously (Bui et al. 2014; 461 Khosravi et al. 2015). However, to our knowledge, studies 462 focused specifically on the assessment of microalgae hydro- 463 lysates for this purpose are not available and even less with 464 regard to *Arthrospira* sp.

465 The dietary inclusion of *Arthrospira* sp. raw biomass in 466 aquafeeds has been evaluated previously in different fish spe- 467 cies with favourable results. Thus, Adel et al. (2016) and Yu 468 et al. (2018) revealed that the incorporation of 10% 469 *Arthrospira* in feeds yielded positive effects on growth perfor- 470 mance in great sturgeon and coral trout (*Plectropomus* 471 *leopardus*), respectively. Similarly, Kim et al. (2013) reported 472 positive effects on fish performance with 5% *Arthrospira* in- 473 clusion level in feeds for parrot fish (*Oplegnathus fasciatus*). 474 However, results obtained in our work revealed that the die- 475 tary inclusion of *Arthrospira* protein hydrolysate up to 4% did 476 not increase fish performance after a 128-day feeding trial.

477 Although some studies have reported the effect of 478 *Arthrospira* in muscle composition of fish, disparate results 479 have been reported. Hence, Velasquez et al. (2016) observed

t3.1 t3.2 t3.3 t3.4 **Table 3** Muscle proximate composition (g kg^{-1} of dry weight) and moisture (%) of gilthead seabream at the end of the feeding trial

	CT				AH-2				AH-4				*p*
Total protein	745.31	±	3.83	a	789.38	±	0.51	c	772.19	±	0.77	b	*0.035*
Total lipid	166.65	±	2.36	c	141.72	±	1.02	a	157.61	±	0.57	b	*<0.001*
Ash	54.06	±	3.32		55.23	±	1.87		54.82	±	2.13		*0.114*
Moisture (%)	74.51	±	0.63		75.67	±	1.02		74.89	±	0.71		*0.7302*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate determination ($n = 3$). Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$)

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Table 4 Changes in skin colour and muscle TBARS content during cold storage in gilthead seabream fed the experimental diets for 128 days

		CT				AH-2				AH-4				*p*
L*	1	76.4	±	1.8	aB	83.3	±	1.9	b	74.8	±	1.3	a	0.009
	8	59.6	±	2.6	aA	77.6	±	2.4	b	76.5	±	1.4	b	< 0.001
	p	*< 0.001*				*0.828*				*0.072*				
a*	1	− 1.3	±	0.4	a	2.3	±	0.2	bB	3.5	±	0.3	cB	< 0.001
	8	− 1.3	±	0.2		1.9	±	0.2	A	1.6	±	0.3	A	0.353
	p	*0.409*				*0.040*				*<0.001*				
b*	1	5.6	±	0.6	B	5.7	±	0.8	A	4.3	±	1.0	A	0.169
	8	2.0	±	0.4	aA	6.7	±	0.6	bB	6.3	±	0.7	bB	< 0.001
	p	*0.002*				*0.027*				*0.030*				
TBARS	1	0.40	±	0.01	bA	0.34	±	0.01	aA	0.34	±	0.02	aA	0.019
	8	0.50	±	0.01	bB	0.44	±	0.01	aB	0.42	±	0.01	aB	0.005
	p	*0.012*				*0.015*				*0.019*				

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE ($n = 9$). Superscript lowercase letters indicate differences ($p < 0.05$) attributable to diets; superscripts capital letters indicate differences attributable to storage time

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no changes in protein, lipid, moisture, or ash contents; on the contrary, our results (Table 3) revealed modifications in muscle protein and lipid contents in fish fed with the diets containing the microalgal protein hydrolysate. Similarly, Roohani et al. (2019) reported that *Arthrospira* (*Spirulina*) *platensis* increased protein and decreased fat content in *Salmo trutta* juveniles. These authors pointed out that the presence of several nutrients (not least vitamins, minerals, essential amino acids, and fatty acids) in microalgae might activate fish metabolism. Chen et al. (2019) found that *Arthrospira* contains substances able to modulate lipid metabolism in rodents, although the chemical nature of those compounds remains to be ascertained. According to Kim et al. (2013), *Arthrospira* might well have certain impact on lipid turnover, mainly through the use of dietary lipids as energy source, this leading to reduced muscle lipid storage. The same effect has been reported previously for other microalgae species (Hussein et al. 2013; El-Sheekh et al. 2014; Vizcaíno et al. 2014, 2016). The dietary inclusion of *Arthrospira* sp. also yields muscle fish with high protein content, an effect that might be highly desirable for final aquaculture products. In agreement, Xu et al. (2018) reported increased muscle protein content as a result of feeding juvenile *Cyprinus carpio* with enzymatic hydrolysates of insect meal. Two hypotheses might explain such increase; on one hand, a raise in the bioavailability and absorption rate of small peptides and free amino acids that enables enhanced body protein synthesis and on the other hand, the increased activity of the digestive enzymes that might promote a more efficient hydrolysis/absorption of nutrients directly involved in the synthesis of tissue protein. Further studies are required in order to verify these hypotheses.

It is a well-known phenomenon that the skin of many species of commercial fish lacks colour and brightness in captivity, a feature directly linked to the consumer's acceptance of fish, which, accordingly, influences their market value. In this regard, different studies have described positive effects of the addition of *Arthrospira* at low inclusion level on the pigmentation attributes in different fish species (Kumprom et al. 2011; Teimouri et al. 2013; Abdulrahman and Ameen 2014; Roohani et al. 2019). Our results indicate that juveniles fed with *Arthrospira*-supplemented diets showed a skin lighter, reddish,

Table 5 Digestive enzyme activities (U g^{-1} tissue) measured in intestine of gilthead seabream juveniles fed experimental diets for 128 days

	CT				AH-2				AH-4				*p*
Total alkaline protease	118.4	±	11.26		95.6	±	2.77		124.3	±	14.72		*0.160*
Trypsin	27.8	±	1.38	a	32.7	±	0.72	b	37.9	±	1.02	c	*0.001*
Chymotrypsin	25.4	±	1.14	a	32.8	±	1.39	b	44.9	±	3.94	c	*0.001*
L-aminopeptidase	0.22	±	0.02	a	0.40	±	0.01	b	0.38	±	0.01	c	*< 0.001*
Alkaline phosphatase	57.2	±	3.77		54.5	±	3.18		54.9	±	3.03		*0.844*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate determinations per tank ($n = 9$). Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$)

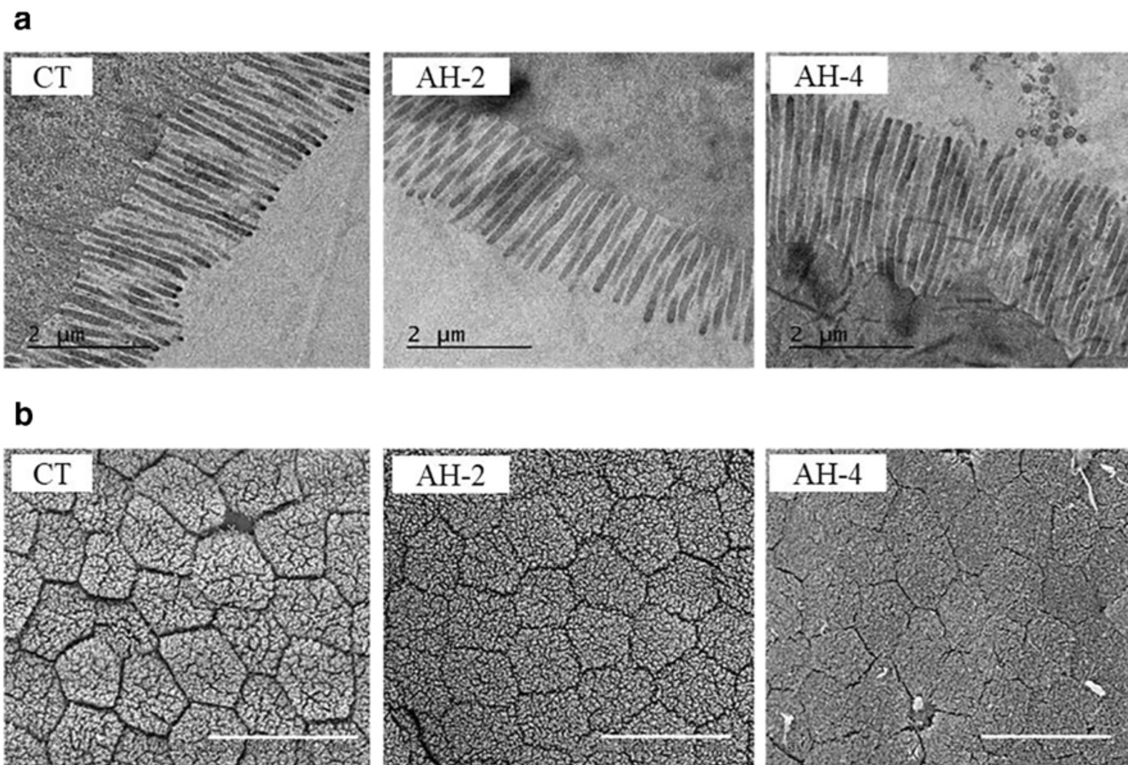

Fig. 3 TEM (**a**) and SEM (**b**) micrographs from the anterior intestine of juvenile gilthead seabream fed with experimental diets (TEM bar, 2 μm; SEM bar, 10 μm). *CT* control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate

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522 and yellowish, and these differences remained stable over 523 8 days of cold storage. Similar results indicating intensified 524 redness and yellowness in fish skin were found in golden carp 525 (*Carassius auratus*; Kumprom et al. 2011), common carp 526 (*Cyprinus carpio*; Abdulrahman and Ameen 2014), and rain- 527 bow trout (Teimouri et al. 2013), fed with *Arthrospira* at a low 528 dietary inclusion level. This improvement in colorimetric pa- 529 rameters could be associated to the fact that most microalgae 530 species are natural sources of pigments (Begum et al. 2016), 531 which might play decisive role on the quality of the final 532 product (Ginés et al. 2004). In this regard, changes in colour 533 parameters observed could likely be attributed to the carotenoid 534 content of *Arthrospira* (Lu et al. 2003; Teimouri et al. 2013), 535 and thus, xanthophylls (mainly lutein and zeaxanthin) could 536 explain the increased yellowness measured in the skin of fillets 537 (Table 4).

538 Muscle lipid oxidation increased during the storage of fish 539 fillets, as evidenced by the significant increase of TBARS. 540 However, values for this parameter in muscle were significant- 541 ly lower in the specimens fed with AH-2 and AH-4 diets 542 (Table 4). The antioxidant capacity of *Arthrospira* sp. is 543 well-known, owing to the high content in different bioactive 544 substances playing a key role in the inhibition of lipid perox- 545 idation (Deng and Chow 2010; Kim et al. 2013). Beyond their 546 influence on colour parameters, xanthophylls have a potent 547 antioxidant capacity against reactive oxygen species (ROS) 548 (Hallerud 2014) that could explain the reduced lipid peroxi- 549 dation of muscle lipids found in those animals fed with AH 550 diets. Moreover, it has also been described that *Arthrospira* sp. 551 contains considerable amount of the enzyme superoxide dis- 552 mutase that might decrease the rate of formation of free radi- 553 cals, this resulting in lower muscle lipid oxidation at inclusion

t6.1 **Table 6** Microvilli morphometric parameters of the anterior intestine of juvenile gilthead seabream fed with the experimental diets for 128 days

		CT				AH-2				AH-4				*p*
t6.3	ML (μm)	1.83	±	0.05	a	1.70	±	0.05	a	2.62	±	0.05	b	*< 0.001*
t6.4	MD (μm)	0.13	±	0.00	a	0.13	±	0.00	a	0.14	±	0.00	b	*0.012*
t6.5	EA (μm^2)	23.36	±	0.82		21.75	±	0.51		21.18	±	1.01		*0.211*
t6.6	TAS (μm^2)	767.90	±	20.28	a	751.45	±	20.85	a	1347.44	±	20.95	b	*< 0.001*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE ($n = 50$). *ML* microvilli length, *MD* microvilli diameter, *EA* enterocyte apical area, *TAS* total enterocyte absorption surface. Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$)

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554 levels from 10 to 2.5% (Teimouri et al. 2016). Similar results
555 were reported in clownfish (*Oplegnathus fasciatus*) (Kim
556 et al. 2013) and in tilapia (Amer 2016), attributed to the inclu-
557 sion of *Arthrospira pacifica* and *Arthrospira* sp. as dietary
558 additives.

559 The activity of digestive enzymes is not only a reli-
560 able indicator of the nutritional status of fish (Cahu and
561 Infante 2001; Cara et al. 2007) but also a valuable tool
562 for estimating the digestive and absorptive capacity of
563 animals after a dietary treatment (Alarcón et al. 1998;
564 Messina et al. 2019). The existence of changes in the
565 digestive-absorptive processes influenced by the dietary
566 inclusion of microalgae has been previously assessed in
567 aquacultured fish such as seabream (Vizcaíno et al. 2014,
568 2016), seabass (Messina et al. 2019), Senegalese sole
569 (Vizcaíno et al. 2018), common carp (*Cyprinus carpio*)
570 (Ansarifard et al. 2018), or great sturgeon (Adel et al.
571 2016). The present study confirmed that dietary inclusion
572 of *Arthrospira* hydrolysates increased the activity of
573 some digestive enzyme activities, despite the low inclu-
574 sion levels tested. Thus, trypsin and chymotrypsin activ-
575 ities increased significantly in fish fed with *Arthrospira*-
576 supplemented diets, a fact that might have contributed to
577 increasing the availability of substrates for muscle pro-
578 tein accretion. Vizcaíno et al. (2016) reported similar
579 results in *Sparus aurata* fed with microalgae-
580 supplemented diets, which might be related to the exis-
581 tence of compensatory mechanisms in fish against die-
582 tary changes. In line with the above, it has been reported
583 that digestive protease and amylase activities increased
584 adding 3% dietary supplementation with plant protein
585 hydrolysate in juvenile blunt snout bream *Megalobrama*
586 *amblycephala* (Yuan et al. 2019). Regarding brush border
587 enzymes, a significant increase in the activity of leucine
588 aminopeptidase was observed in fish fed with the diets
589 containing the microalgal hydrolysate (Table 5). Leucine
590 aminopeptidase and alkaline phosphatase play a crucial
591 role in the final stages of the digestive process, facilitat-
592 ing the absorption and transport of nutrients through the
593 enterocytes (Infante and Cahu 2001). In fact, both en-
594 zymes are used as indicators of the intestinal integrity
595 (Wahnon et al. 1992) or as general markers of nutrient
596 absorption (Silva et al. 2010). Previous studies proposed
597 that the higher the activity levels of these enzymes, the
598 better the efficiency of the digestive processes and the
599 intestinal absorptive capacity (Infante and Cahu 2001).
600 However, Messina et al. (2019) reported that alkaline
601 phosphatase activity was not affected when fishmeal
602 was replaced by microalgae, indicating no major func-
603 tional changes in the gut integrity of European seabass
604 (*Dicentrarchus labrax*).

605 In addition to the activity of the digestive enzymes, the
606 intestinal mucosa plays a key role in the digestive and
607 absorptive processes (Sweetman et al. 2008), as well as acting
608 as a protective barrier against pathogenic microorganisms
609 (Wilson and Castro 2011). The study of the intestinal mucosa
610 also enables to know the influence of dietary treatments on its
611 structure and morphology. Several studies revealed that the
612 dietary inclusion of plant protein ingredients, algae, or
613 probiotics can lead to morphological changes in the structure
614 of the digestive mucosa, which are linked to important conse-
615 quences on the digestive physiology and the absorption ca-
616 pacity of the intestinal mucosa. This has been described in
617 different fish species, such as gilthead seabream (Cerezuela
618 et al. 2012; Vizcaíno et al. 2016), rainbow trout (Araújo et al.
619 2016), goldfish (*Carassius auratus*) (Omnes et al. 2015), or
620 Senegalese sole (Vizcaíno et al. 2018). Until now, knowledge
621 regarding the effects of protein hydrolysates on the intestinal
622 structure is scarce. In the present study, the ultrastructural
623 analysis revealed a significant effect of *Arthrospira* hydroly-
624 sate on the morphology of the intestinal mucosa, especially in
625 those fish fed with 4% inclusion level. In agreement, it has
626 been described that the dietary inclusion of microalgal hydro-
627 lysates can reduce mucosal barrier damage, as well as prevents
628 colonic inflammation in mice (Wang et al. 2018). These au-
629 thors evidenced that the oral administration of microalgae hy-
630 drolysates reversed the progression of dextran sulphate
631 sodium-induced colitis and also prevented acute inflammation
632 in that murine model. In agreement, the inclusion of 5% die-
633 tary shrimp hydrolysate resulted in larger intestinal villi and
634 also modulated the transcriptomic response of the intestinal
635 mucosa in European seabass (Leduc et al. 2018). Yuan et al.
636 (2019) reported that 3% cottonseed meal protein hydrolysate
637 increased the length of the intestinal microvilli in juvenile
638 blunt snout bream (*Megalobrama amblycephala*). In our
639 study, changes observed on microvilli length and microvilli
640 diameter can be interpreted as an overall increase in enterocyte
641 absorption surface and, consequently, an enhanced intestinal
642 absorption capacity. This increased absorption area might
643 have been responsible for higher amino acid uptake in the
644 anterior intestine, this yielding higher protein accretion in
645 muscle, especially in fish fed with 4% microalgae hydrolysate.

646 In conclusion, our results show that juvenile gilthead
647 seabream fed with *Arthrospira* hydrolysate increased the ac-
648 tivity of key digestive enzymes, improved the intestinal mu-
649 cosa structure, and reduced the oxidation of muscle lipids.
650 Thus, this supplement (especially when used at 4% inclusion
651 level) could be useful for maintaining the overall condition
652 status in juveniles of this fish species. The incorporation of
653 microalgal hydrolysate as dietary additive seems promising
654 for feeding *S. aurata* juveniles, not least due to the stimulating
655 effect observed on the intestinal mucosa and as a natural al-
656 ternative for the improvement of the skin colour in cultured
657 fish. However, future studies should be focused on the intrin-
658 sic mechanism of their effects, as well as on the feasibility of
659 its commercial use in aquafeeds at large scale.

Authors' contributions A. Galafat and A.J. Vizcaíno performed the fish sampling. I. Jérez-Cepa and J.M. Mancera participated in sampling and fish maintenance. A. Galafat, A.J. Vizcaíno, and M.I Sáez performed analytical analysis. F.J. Alarcón prepared the aquafeeds. A. Galafat, F.J. Alarcón, and A.J Vizcaíno performed the data analysis and drafted the manuscript. F.J. Alarcón and T.F. Martínez designed the work. 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 UCA (Universidad de Cádiz, Spain).

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