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Effects of Micro- and Macroalgae-Supplemented Diets on Growth and Muscle Fibrillar Constitution of Gilthead Seabream, *Sparus aurata* L., in the Final On-Growing Phase

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Abstract: The influence of algae in a final fattening diet for gilthead seabream, *Sparus aurata*, was studied in an 87 d feeding trial. Five groups were analysed (three tanks per group). In the first 38 days, one lot was fed with standard algae-free feed (C1) and four batches were fed with an algae-free diet low in fishmeal (FM) and fish oil (FO) and rich in terrestrial vegetables (C2). Subsequently, the C2 diet was changed in three out of the four groups, two of them being fed a diet with a microalgae blend (10%) plus 2% macroalgae (*Alaria esculenta*), either in raw (C2-R) or hydrolysed (C2-H) form, and a third batch with a diet lacking FM and FO but supplemented with 10% microalgae and 7% algae oil (C2-O) for 49 days. Body length and weight, muscle parameters, and conversion index were analysed after 38 and 87 days. At day 38, no significant differences were observed in any parameter between C1 and C2, but at the end of the trial (day 87), C2 displayed the lowest weight gain and the worst conversion rate, whereas C2-R showed higher body weight, fibrillar hypertrophy, and better conversion rates than the other groups, and C2-H displayed higher hyperplasia values than the other groups.

Keywords: microalgae blend; *Alaria esculenta*; fattening feed; muscle fibre hyperplasia

Key Contribution: The results showed that the inclusion of algae in the final fattening diet of *Sparus aurata* can partially replace fish meal (FM) and fish oil (FO). This fact allows a reduction in the excess of terrestrial vegetables that are normally used as partial substitutes for FM and FO. Microalgae hydrolysis generated the highest number of muscle fibres in the fillet, which is usually correlated with higher fillet firmness values.

Academic Editors: Houguo Xu and Qiang Ma

Received: 6 May 2025
Revised: 23 May 2025
Accepted: 26 May 2025
Published: 2 June 2025

Citation: Ayala, M.D.; Chaves-Pozo, E.; Sáez, M.I.; Alarcón, F.J.; Martínez, T.F.; Arizcun, M. Effects of Micro- and Macroalgae-Supplemented Diets on Growth and Muscle Fibrillar Constitution of Gilthead Seabream, *Sparus aurata* L., in the Final On-Growing Phase. *Fishes* **2025**, *10*, 262. https://doi.org/10.3390/fishes10060262

1. Introduction

Currently, the feeds that are used in the aquaculture industry for feeding fish include a high percentage of plant-based ingredients to partially replace fishmeal and fish oil, thus reducing production costs [1]. However, ingredient from vegetables sources have some disadvantages such as unbalanced amino acid composition and n-3 polyunsaturated fatty acid (PUFA) deficiency, amongst others [1,2]. This can trigger lower fish growth and lower quality of fillets for human consumption [3]. In this framework, microalgae are a valuable

alternative to the excessive use of vegetables due to their high protein content and their balanced profile of essential amino acids and n-3 PUFAs [1,4]. In addition, microalgae are beneficial for the fishes' growth and health [1]. Several studies have shown a positive effect of microalgae on fish growth within a wide range of parameters. Favourable fish growth has been observed when *Arthrospira* sp. has been used as a substitute for fishmeal in the range of 5 to 50% in different fish species [5–9]. Interestingly, lower inclusion levels of *Arthrospira platensis* also increase weight gain and specific growth rates in juvenile Caspian brown trout (*Salmo trutta caspius*) [10]. The microalga *Chlorella vulgaris* as a partial substitute for fishmeal in range from 5 to 25% showed a clear positive correlation between the inclusion levels and the weight gain, protein efficiency index, daily feed intake, and specific growth rate in several fish species [11,12]. However, in juvenile zebrafish (*Danio rerio*) the maximum values of weight gain and growth rate were achieved with 4% level of fishmeal substitution by *Chlorella* meal [13], while higher values up to 25% inclusion levels showed detrimental effects on fish growth [14]. These results, all together, indicate that microalgae can replace fishmeal in the diet of fish, but the optimal levels of inclusion depend on fish and microalgae species, with low or medium inclusion levels being preferable in terms of fish growth. So, obtaining universal conclusions is not feasible, and a species-specific assessment is therefore mandatory for using microalgae as feed ingredient. On the other hand, it is also important to consider the cost of microalgae production. In this regard, it should be noted that incorporating microalgae at high levels is costly. However, it has been observed that incorporating them as functional additives at low levels can be sufficient to promote growth and improve general aspects of fish physiology [15,16]. Therefore, research is currently focused on studying the effect at low inclusion levels.

Microalgae are also considered as an alternative to fish oil, as they can synthesize docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or both. In fact, microalgae are the most relevant primary natural producers of n-3 PUFAs in marine ecosystems. Interestingly, microalgae PUFA supplementation in diet also promotes growth and enhances the immune response of aquatic animals [17,18]. Microalgae strains and species that can be used to enhance PUFA accumulation in aquatic animals include *Pavlova viridis*, *Scenedesmus* sp., *Nannochloropsis* sp., *Lobosphaera incisa*, *Tribonema ultriculosum*, *Schizochytrium* sp. *Crypthecodinium cohnii*, and *Chlorella* sp. [1]. Thus, *Thraustochytrid schizochytrium* oils replaced fish oil in the diet of salmon (*Salmo salar* L.), with no negative effect on their growth [19]. Other authors [20] carried out the inclusion of *Schizochytrium limacinum* in salmon diets at levels of 2.62–6.25%. Fish supplemented with *S. limacinum* grew significantly, reaching a higher body weight at the end of the experiment, but had a feed conversion ratio, survival rate, and biometric indices similar to the group fed with fish oil. Fish treated with *S. limacinum* contained higher concentrations of EPA and DHA in the fillet. Qiao et al. [21] fed juvenile plaice (*Paralichthys olivaceus*) with levels ranging from 4.56 to 13.6% of *Schizochytrium* and *Nannochloropsis* and obtained similar values to those obtained with fish oil in terms of total body composition, liver and muscle lipid content, and plasma lipid composition. Also, in gilthead seabream (*Sparus aurata*), dehydrated biomass of *Schizochytrium* sp. or *Crypthecodinium cohnii* showed similar larval growth and survival rates to fish fed with fish oil [22–24].

On the other hand, the diet also influences the muscle growth mechanisms, which are hyperplasia (generation of new muscle fibres) and hypertrophy (increase in the size of muscle fibres) [25,26]. Both mechanisms influence the quality of the fish fillet, mainly the texture. Thus, it has been observed that a greater number of fibres (fibrillar hyperplasia) is correlated with a greater firmness of the fillet [27,28]. There are very few studies on the influence that microalgae supplementation in the diet has on the fibrillar constitution of fish muscle. Knutsen et al. [29] studied the influence of *Nannochloropsis oceanica* in

the diet of the spotted wolffish (*Anarhichas minor*) and found no differences in muscle growth or in its fibrillar constitution. Recently, the influence of *N. gaditana* was studied in the final fattening of sea bream [30,31]; the authors observed that microalgae had a positive effect on the firmness of the fillet, and this was correlated with the density of white muscle fibres. However, there is very little scientific literature on this subject, so further studies are needed to delve deeper into the matter. In the aforementioned studies carried out during the final fattening of sea bream [30,31], the authors only used one species of microalgae: *Nannochloropsis gaditana*. In the present work, we have used a blend of microalgae: *Chlorella vulgaris*, *Nannochloropsis gaditana*, *Arthrospira platensis*, *Schizochytrium* sp., and *Dunaliella salina*. This combination of microalgae constitutes a mixture rich in proteins and essential amino acids, as well as polyunsaturated fatty acids, such as DHA and EPA. This mixture has already been used previously by our research team during the larval phase of gilthead sea bream, with good results in its growth [32]. Furthermore, a marine brown macroalgae species, *Alaria esculenta*, was also included as a dietary ingredient in the present work. Macroalgae biomass has a relatively high protein quality compared to cereal and soybean meal, and a low lipid content (1–5%), but most of them are PUFAs. However, its potential as a feed macro ingredient for carnivorous commercial Mediterranean fish species is scarce owing to their richness in structural carbohydrates [33]. Seaweeds are very rich in secondary metabolites (pigments, carotenoids, etc.) and minerals (iodine, zinc, etc.), with valuable bioactive effects on the physiology of farmed fish, and have been little studied as an additive in fish feed.

Considering the studies carried out so far, in the present work, we have used a mixture of microalgae with complementary properties at 10% inclusion levels, as well as a macroalga (*Alaria esculenta*), as a feed additive at low doses (2%) in the final fattening phase of the sea bream up to commercial size. These algae were used as a partial replacement for fish and vegetable meal, as well as fish oil. The algae used in the present work were used in two forms: raw and hydrolysed, the latter in order to improve the digestibility of microalgae that have thick cell walls, such as *Nannochloropsis* and *Chlorella* [1,30,34]. In the present study, we have used a mixture of enzymes, most of which have been previously used in sea bream fed with *Nannochloropsis gaditana* [30,31], and other species, such as in rainbow trout (*Oncorhynchus mykiss*) fed with *Nannochloropsis oculata* [35], showing an improvement in digestibility in the cited studies.

2. Material and Methods

2.1. Animals and Rearing Conditions

This experiment was carried out at the experimental marine culture facilities of the Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (COMU-IEO), CSIC. Two-year old gilthead seabream (293.8 ± 1.4 g average initial body weight and 26.9 ± 0.05 cm average total length), born and bred at COMU-IEO facilities, were randomly distributed in 15 tanks of 2000 L (21 fish tank^{-1}), with an initial culture density of 3 Kg m^{-3}. These 15 tanks corresponded to 5 experimental groups (3 tanks feeding group^{-1}) fed with different diets (isonitrogenous and isolipidic) (Table 1). Therefore, the total number of fish per group was 63. The diets were prepared at the Experimental Feed Service of the University of Almería (https://www.ual.es/universidad/serviciosgenerales/stecnicos/perifericos-convenio/piensos-experimentales) accessed on 3 March 2023:

- Group C1: fish fed with standard algae-free feed containing 15% fishmeal (FM) and 10% fish oil (FO) throughout the experiment (87 days).
- Group C2: fed with an algae-free diet, low in FM (5%) and FO (5%) and rich in vegetable sources (soybean meal, wheat flour and gluten, soybean oil and rapeseed oil), throughout the experiment (87 days).

- Group C2-R: fed for 38 days with diet C2 and then transferred for 49 days to a diet containing 10% of a mixture of raw microalgae (*Chlorella vulgaris*, *Nannochloropsis gaditana*, *Arthrospira platensis*, *Schizochytrium* sp., and *Dunaliella salina*) and 2% of the macroalga Alaria esculenta, replacing the 5% FM, 0.7% FO, and 6.4% terrestrial vegetables of the standard C1 diet.
- Group C2-H: like C2-R, but the algal biomass (10% microalgae and 2% macroalgae) was subjected to fibrolytic enzymatic hydrolysis with cellulase and β-glucanase activities to cause the rupture of cell walls and promote the assimilation of intracellular nutrients. Proteolytic enzymes (endo- and exopeptidases) (Novozymes®, DK) were also used to convert high molecular weight proteins into low-molecular-weight hydrolysates. The enzyme cocktail was added to the microalgae biomass under agitation.
- Group C2-O: after 38 days on diet C2, the group was switched for an additional 49 days to a diet lacking FM and FO, supplemented with the above-mentioned raw microalgae mixture at 10% replacing the FM and terrestrial vegetables of the standard C1 diet. Also, 7% algae oil was added to this diet to replace the 7% FO of the standard C1 diet.

As can be seen, groups C1 and C2 were fed the same diet throughout the experiment (from day 1 to day 87). However, the other three groups (C2-R, C2-H, and C2-0) were maintained under the same conditions as C2 for the first 38 days, and their diets were subsequently modified until day 87 of the experiment to determine whether the inclusion of algae in these groups could positively influence their growth.

Fish were fed by hand 3 times a day (9:00, 14:00 and 18:00 h) to apparent satiation, up to a maximum of 1.3% feeding rate (1.3% of the weight of fish in the tank). Animals were kept under 12L:12D photoperiod and natural temperature. Thus, the water temperature increased gradually from 14 °C at the beginning to 21 °C at the end of the feeding trial. The salinity of the water was 38 parts per thousand (ppt). Tanks were equipped with aerators to maintain a level of oxygenation above 6 mg L^{-1}. In parallel, oxygen was supplied to the tanks when necessary. The oxygen level was measured with sensors from OxyGuard International A/S, Denmark. Nitrite and ammonium levels were measured with commercial kits (SERA® GmbH, Heinsberg, Germany). The levels of nitrate and ammonia were monitored daily and always kept at the optimal levels for gilthead seabream culture (<0.1 mg L^{-1}).

Table 1. Ingredients and proximal composition (% dry matter) of the five experimental diets.

Ingredients	C1	C2	C2-R	C2-H	C2-O
Fishmeal LT94 [1]	15.00	5.00	10.00	10.00	
Poultry meal [2]					5.00
Lysine [3]	0.80	1.60	0.80	0.80	1.60
Metionina [4]	0.30	0.60	0.30	0.30	0.80
Squid meal [5]	2.00	0.25	2.00	2.00	
Fish meal hydrolysate CPSP90 [6]	1.00	0.25	1.00	1.00	
Krill meal [7]	1.00	0.25	1.00	1.00	
Blood meal [8]					2.00
Tenebrio molitor meal [9]					5.00
Hermetia illucens meal [10]					5.00
Microalgal blend [11]			10.00	10.00	10.00
Alaria esculenta meal [12]			2.00	2.00	
Wheat gluten [13]	16.00	18.00	16.00	16.00	15.00
Soybean meal [14]	16.00	24.00	15.00	15.00	8.30

Table 1. *Cont.*

Ingredients	C1	C2	C2-R	C2-H	C2-O
Soybean protein concentrate [15]	11.00	17.00	11.10	11.10	8.20
Pea protein concentrate [16]					5.00
Fish oil [17]	10.00	5.00	9.30	9.30	
Algal oil [18]					7.00
Soybean and rapeseed oil [19]	4.00	10.50	4.00	4.00	5.90
Soybean lecithin [20]	1.00	1.00	1.00	1.00	1.00
Wheat meal [21]	18.80	13.45	13.40	13.40	17.10
Choline chloride [22]	0.50	0.50	0.50	0.50	0.50
Betaine [23]	0.50	0.50	0.50	0.50	0.50
Vitamin and mineral premix [24]	2.00	2.00	2.00	2.00	2.00
Vitamin C [25]	0.10	0.10	0.10	0.10	0.10
Proximate composition					
Crude protein	47.8	47.9	47.1	47.0	47.7
Crude lipid	18.2	18.4	18.8	18.8	18.6
Ash	7.1	5.8	8.4	8.4	6.4
Carbohydrates	27.0	27.9	25.7	25.8	27.3
Moisture	4.7	4.8	4.8	4.7	4.7

[1] 69.4% crude protein, 12.3% crude lipid (Norsildemel, Bergen, Norway). [2] Aquatract sol 70% crude protein, 9% crude lipid (Gepro, Germany). [3,4] Lorca Nutrición Animal SA (Murcia, Spain). [5,6,7] purchased from Bacarel (UK). CPSP90 is enzymatically pre-digested fishmeal. [8] Common baits (Germany). [9] Defatted *Tenebrio* meal (Ynsect, France). [10] Defatted *Hermetia* meal (Protix, The Netherlands). [11] Microalgal blend consisting of *Chlorella vulgaris*, *Nannochloropsis gaditana*, *Arthrospira platensis*, *Schizochytrium* sp., and *Dunaliella salina*. [12] *Alaria esculenta* biomass provided by Bantry Marine Research Station (Ireland). [13] 78% crude protein (Lorca Nutrición Animal SA, Murcia, Spain). [14] Soycomil, 60% crude protein, 1.5% crude lipid (ADM, Poland). [15] 50% crude protein (Lorca Nutrición Animal SA, Murcia, Spain). [16] Pea protein concentrate, 85% crude protein, 1.5% crude lipid (Emilio Peña SA, Spain). [17] AF117DHA (Afamsa, Spain). [18] Veramaris oil. [19] Soybean oil and rapeseed oil (1:1) (Aceites el Niño, Spain). [20] P700IP (Lecico, DE). [21] Local provider (Almería, Spain). [22,23] Andres Pintaluba, Spain. [24] *Life bioencapsulation* SL (Almería, Spain). Vitamins (mg kg^{-1}): vitamin A (retinylacetate), 2,000,000 UI; vitamin D3 (DL-cholecalciferol), 200,000 UI; vitamin E (Lutavit E50), 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2500 mg; vitamin B1 (thiamine hydrochloride), 3000 mg; vitamin B2 (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 (Betafin S1), 50,000 mg. Minerals (mg kg^{-1}): 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), 18.6%; (186,000 mg); KCl, 2.41%; (24,100 mg); NaCl, 4.0% (40,000 mg). [25] TECNOVIT, Spain.

2.2. Algal Biomass Pre-Treatment

Enzymatic hydrolysis was carried out by mixing the algal biomass (microalgae blend plus *Alaria esculenta*) at a final concentration of 150 g dry weight L^{-1} in 50 mM sodium citrate buffer solution (pH 5.5) and incubating at 45 °C under continuous agitation for 5 h, as described by Sáez et al. [36]. Proteolytic (Alcalase®, Novozymes) and fibrolytic (cellulase and β-glucanase, Sigma Aldrich) enzymes were added at an enzyme-to-algae ratio of 0.05 (50 g enzyme mixture kg^{-1} dry algal blend). Following the hydrolysis, the mixture was immediately used for manufacturing aquafeeds.

2.3. Experimental Diets

Table 1 shows the composition of the five experimental diets, which were isonitrogenous and isolipidic. The diets were prepared at the Experimental Feed Service of the University of Almería (https://www.ual.es/universidad/serviciosgenerales/stecnicos/perifericos-convenio/piensos-experimentales) accessed on 3 March 2023. The ingredients were ground and mixed in a vertical helical mixer (Sammic 1 M-11, 5 L capacity, Sammic SA, Azpeitia, Spain) for 20 min. Subsequently, the algae (raw or hydrolysed) were added, and the water content was adjusted to obtain 400 mL kg^{-1} of the ingredient mixture, obtaining a homogeneous mass, which was passed through a single-screw laboratory extruder

(Miltenz 51SP, JS Conwell Ltd., Taupo, New Zealand) equipped with dies to obtain 4 mm pellets. Feeds were dried under forced air circulation (Air frio, Almería, Spain) at 0 °C for 24 h and subsequently stored at −20 °C. Proximate analysis (dry matter, ash, and crude protein, N × 6.25) of feeds samples were determined according to AOAC procedures [37]. Lipids were extracted following the methodology proposed by Folch [38], using chloroform/methanol (2:1 *v*/*v*) as a solvent, and total lipid content was calculated gravimetrically.

2.4. Sampling Points

Sampling was carried out on day 38 of the experiment in groups C1 and C2. At the end of the experiment (day 87 of the experiment), sampling was carried out in all experimental groups (C1, C2, C2-R, C2-H, C2-O).

2.5. Body Parameters, Conversion Rates, Specific Growth Rate and Survival

In each sampling, the body length and weight of 30 fish per group were measured. To do this, fish were anesthetized (40 µL L^{-1} clove oil in sea water) and then individually recorded. At the end of the experiment, conversion rates (feed consumed weight $gain^{-1}$), specific growth rate (grams day^{-1}) and survival rates were calculated in all groups.

2.6. Muscle Growth

For muscle analysis, nine specimens per group (three fish per $tank^{-1}$) were sacrificed by anaesthesia overdose (60 µL L^{-1} of clove oil in seawater) and then transported on ice to the Faculty of Veterinary Medicine, University of Murcia. The fish were cut transversely at the anal opening. Subsequently, the complete transverse muscle section of each fish was photographed for morphometric analysis (Sygma-Scan Pro_5 system, Systat Version 5.0 Inc., San Jose, CA, USA) following the methodology described in previous works [30]. Subsequently, 5 mm thick muscle sections were made, which were then divided into smaller blocks. These muscle blocks were frozen in 2-methylbutane over liquid nitrogen. From these blocks, 8 µm thick sections were obtained in a cryostat (Leyca CM 1850, Leica Microsistemas SLU, Barcelona, Spain), which were stained with hematoxylin-eosin for the morphometric study of the following muscle parameters: total cross-sectional area of the white muscle, number of white muscle fibres, area and minor axis length of white muscle fibres, and muscle fibre density (number of white fibres mm^{-2}). The average size of the white muscle fibres of each specimen was estimated from ~600 fibres (±10 SD) located at the intermediate and the apical sectors of the epaxial quadrant of the transversal section of the myotome, according to the methodology described in previous studies in teleosts [30].

2.7. Statistical Analysis

The statistical package SPSS 28 (IBM, New York, NY, USA) was used for the statistical analysis. The Shapiro–Wilk and Levene tests were used to study the data distribution and homogeneity of variances, respectively, for $p < 0.05$. Both tests showed p values > 0.05 for most parameters, and therefore, analysis of variance (ANOVA) and a post hoc Tuckey test were used for $p < 0.05$. However, in cases where p values < 0.05 were obtained in the Shapiro–Wilk test, non-parametric tests (Mann–Whitney U and Kolmogorov–Smirnov Z) were used. All the data were expressed as mean ± standard error (SEM).

3. Results

3.1. Day 38 of the Experiment

Figure 1A,B shows the myotome of groups C1 and C2. The mosaic of fibrillar sizes—typical of adult teleosts—can be observed in both groups, with new small fibres interposed between the large fibrillar fibres.

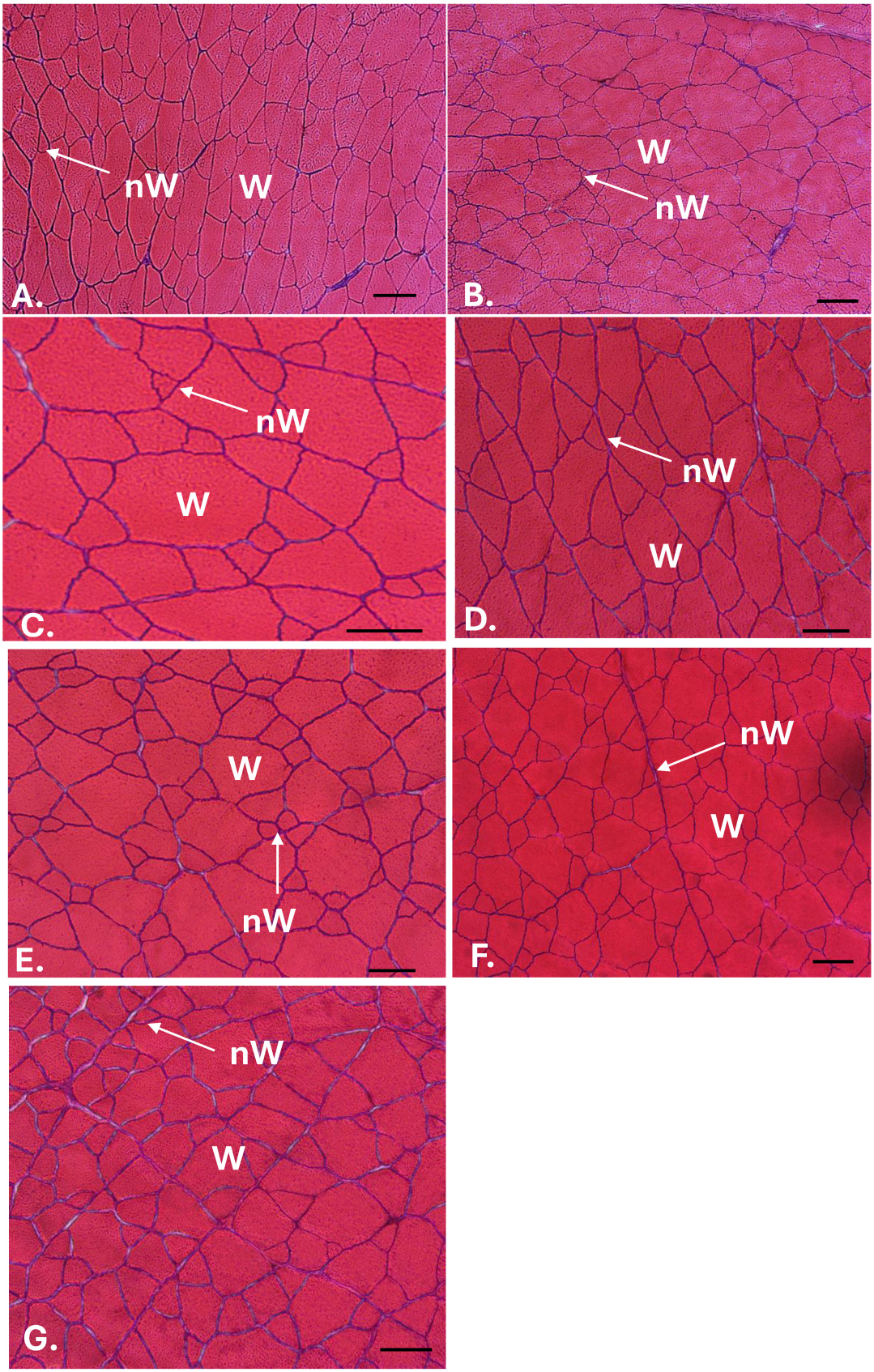

Figure 1. Cross section of the white muscle of gilthead sea breams from groups C1 (**A**) and C2 (**B**) after 38 days of the experiment and from groups C1 (**C**), C2 (**D**), C2-R (**E**), C2-H (**F**), and C2-O (**G**) after 87 days of feeding. Hematoxylin-eosin staining. W: white fibres; nW: new white fibres. Bars 500 µm.

Regarding biometric and muscle parameters (Table 2), no significant differences were observed in any parameter between C1 and C2 groups after 38 days of feeding.

Table 2. Average values (mean ± SEM) of the biometric and muscle parameters measured in C1 and C2 groups after 38 days of the feeding trial. B: cross-sectional area of the white muscle. BL: body length; BW: body weight; A: area of the white fibres. D: minimum diameter of the white fibres; N: number of white fibres; Density: fibrillar density (number of fibres mm^{-2}). Different superscripts in each row indicate significant differences ($p < 0.05$) between the groups, for each parameter. Body and muscle data were obtained from nine specimens per experimental group.

Day 38 of the Experiment		
Groups	C1	C2
BL (cm)	27.0 ± 0.43 ^{a}	26.689 ± 0.25 ^{a}
BW (g)	330.0 ± 12.67 ^{a}	318 ± 11.35 ^{a}
B (cm^2)	19.15 ± 0.94 ^{a}	19.52 ± 0.99 ^{a}
A (μm^2)	7288.0 ± 382.87 ^{a}	6286.22 ± 544.71 ^{a}
D (μm)	87.23 ± 2.14 ^{a}	81.74 ± 3.8 ^{a}
N ($\times 10^3$)	273.34 ± 25.20 ^{a}	307.57 ± 26.97 ^{a}
Density	139.31 ± 8.05 ^{a}	163.37 ± 16.58 ^{a}

3.2. Day 87 of the Experiment

Table 3 and Figure 2 show the biometric and muscle parameters for all groups. The muscle showed the fibrillar mosaic typical of adult teleost in all specimens (Figure 1C–G). As shown in Table 3, the body length was similar in all groups. The highest body weight and specific growth rate values were recorded in the C2-R group, while the lowest values of these parameters were found in the C2 group (Table 3). Interestingly, the C2 group also recorded the worst conversion index (Table 3).

Table 3. Mean values ± SEM of body and muscle data for all groups of sea bream specimens at 87 days of the experiment. BL: body length; BW: body weight; B: cross-sectional area of white muscle; D: minimum diameter of white fibres; Density: fibrillar density (number of fibres mm^{-2}); Conversion rate: feed consumed weight $gain^{-1}$. Specific Growth Rate (SGR) (grams day^{-1}). Different superscripts in each row indicate significant differences ($p < 0.05$) between groups, for each parameter. Body data were obtained from 30 animals per experimental group. Muscle data were obtained from nine specimens per batch.

Day 87 of the Experiment					
Groups	C1	C2	C2-R	C2-H	C2-O
BL (cm)	28.28 ± 0.17 ^{a}	28.24 ± 0.14 ^{a}	28.8 ± 0.25 ^{a}	28.24 ± 0.19 ^{a}	28.35 ± 0.21 ^{a}
BW (g)	378.49 ± 7.68 ^{ab}	355.99 ± 6.88 ^{b}	391.85 ± 6.94 ^{a}	366.83 ± 8.74 ^{ab}	367.22 ± 6.53 ^{ab}
SGR	0.99	0.77	1.51	1.00	1.00
Conversion rate	2.5	3.1	2.3	2.7	2.7
B (cm^2)	24.06 ± 0.9 ^{a}	24.73 ± 0.1 ^{a}	25.04 ± 0.76 ^{a}	25.26 ± 0.16 ^{a}	22.82 ± 0.96 ^{a}
D (μm)	67.76 ± 0.58 ^{a}	66.64 ± 0.64 ^{a}	73.63 ± 0.66 ^{b}	69.44 ± 0.62 ^{a}	66.93 ± 0.61 ^{a}
Density	154.30 ± 9.9 ^{a}	153.09 ± 17.0 ^{a}	126.4 ± 4.72 ^{a}	151.80 ± 6.67 ^{a}	152.69 ± 11.34 ^{a}

The size of the fibres (area and fibrillar diameter) was greater in C2-R than in the other groups ($p < 0.05$) (Table 3; Figure 2), while the highest number of fibres (hyperplasia) was reached in C2-H ($p > 0.05$) (Figure 2).

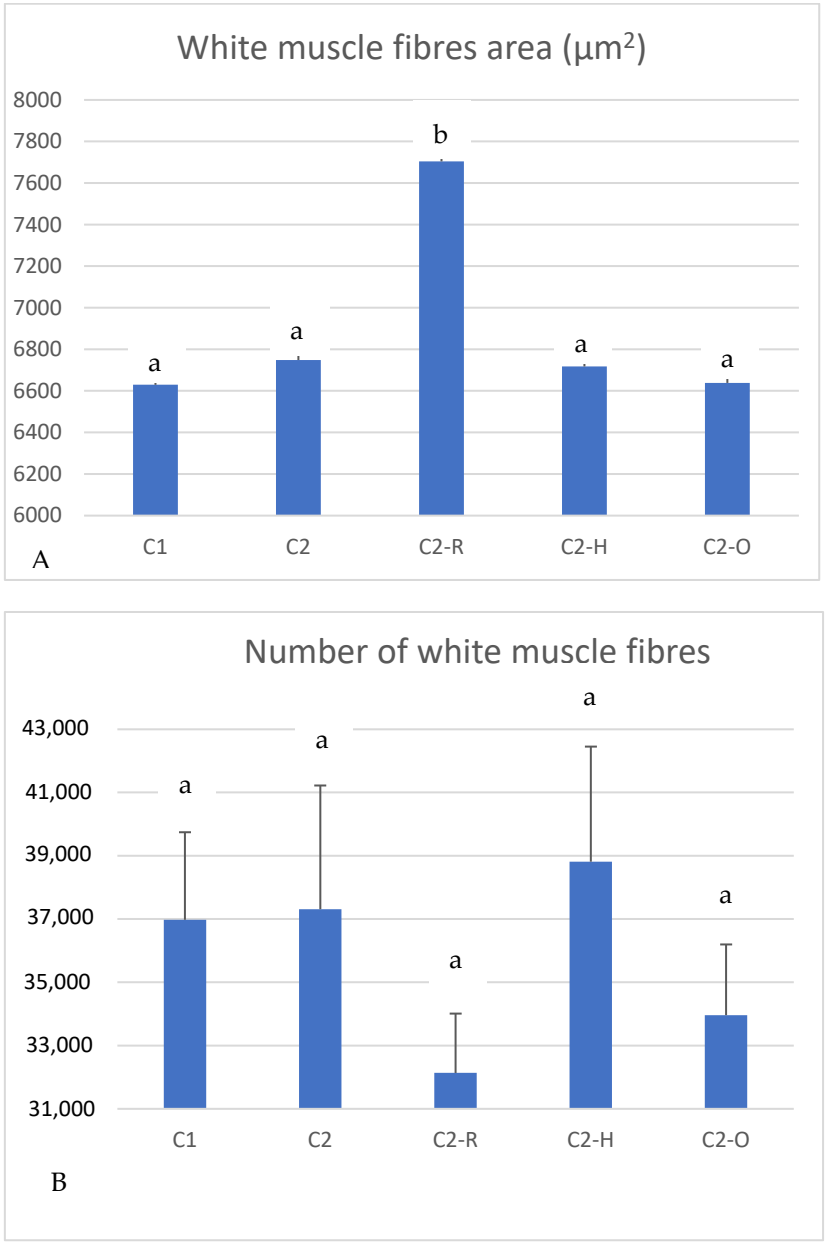

Figure 2. Area (**A**) and number (**B**) of white fibres at the end of the experiment (87 days) in all groups. Different letters on the columns indicate significant differences between groups ($p < 0.05$).

3.3. Survival

Survival was 100% in all the groups at the end of the experiment.

4. Discussion

Many microalgae species have a high protein content and a valuable amino acid profile [1,39]. Thus, some studies have shown that the protein content of *Scenedesmus* sp., *Chlorella* sp., *Dunaliella* sp., and *Arthrospira*, was in the range of 50 to 65% [40]. Therefore, they are presented as a potential alternative to the excessive use of plant-origin ingredients that are currently used to replace FM in commercial aquafeeds. In addition, microalgae have been suggested as an interesting alternative to FO, as they can synthesize DHA, EPA, or both. However, the high cost of the microalgal biomass remains a serious obstacle to its massive incorporation in animal feeds. For this reason, in recent years, the incorporation of algal biomass at low levels in feed (below 10%) is being explored more as an additive than

as an ingredient. The literature indicates that even small amounts in feeds are sufficient to produce a good balance of nutrients in the diet and adequate fish growth [31]. On the other hand, macroalgae contain polyunsaturated fatty acids, pigments, carotenoids, minerals, and proteins with a relatively high quality compared to that contained in soybean and cereal flour [33]. In the present work, a mixture of 10% microalgae and a 2% macroalga (*Alaria esculenta*) was used in raw and hydrolysed diets (C2-R and C2-H, respectively) to partially replace a percentage of FM, FO, and terrestrial vegetables. The results were compared with those obtained with two algae-free diets: a standard diet (C1) and a diet low in FM and FO and with a high percentage of vegetables (C2). Also, the microalgae mixture and algal oil were used in another diet (C2-O) to totally replace FM and FO. After 38 days of feeding sea bream with C1 and C2 diets, no significant differences were observed in any parameter between both groups. At the end of the experiment (day 87), the highest values of body weight and fibrillar hypertrophy were observed in the group supplemented with raw algae (C2-R), which also presented the best values for feed conversion ratio and SGR, whereas the C2 group showed the lowest weight and SGR values and the worst conversion index. On the other hand, all the experimental groups, except for C2, showed body weight and conversion rate values like C1, evidencing that the algae used in the present experiment can partially replace FM, terrestrial vegetables, and FO and reverse the negative effects of the C2 diet on these parameters. Hence, the results of the present work show that low or intermediate levels of algae inclusion in the diet are sufficient to obtain adequate growth in *S. aurata*, which is in line with what has been observed in other species. Thus, in rainbow trout (*Oncorhynchus mykiss*) and three-spot gourami (*Trichopodustri chopterus*), fishmeal substitution with *Arthrospira* at 7.5 and 10% levels produced the best growth, pigmentation, and survival results [8,41]. In juvenile Caspian brown trout, the inclusion of this microalga at 1.32 and 5.3% levels increased weight gain and specific growth rates [10], and in zebrafish juveniles, the highest values of weight gain and growth rate were achieved with replacement levels of 4% of FM by *Chlorella* meal [13].

On the other hand, the highest values of hyperplasia were reached in the group supplemented with hydrolysed algae (C2-H), which seems to indicate that hydrolysis allowed a more efficient use of the nutrients of the algae, since the generation of fibres requires more energy than hypertrophy [42]. Ayala et al. [34] also observed greater hyperplasia in commercial-sized sea bream fed hydrolysed *Nannochloropsis gaditana* in their juvenile phase than fish receiving raw microalgae. Several studies have shown a positive correlation between hyperplasia and fillet firmness [27,28]. Similarly, in previous works, our research team [30,31] studied commercial-sized gilthead sea bream supplemented with 2.5 and 5% *N. gaditana* and found a positive correlation between muscle fibre density and fillet textural firmness. Hence, it is possible that the C2-H group presents higher values of fillet textural firmness than the rest of the groups. To test this, our research team is currently carrying out textural analyses of all the groups included in this work.

Regarding the C2-O group, this showed similar results to those observed in the other groups (even with better conversion levels than those observed in C2), thus showing that this diet can also be a good alternative to reverse the negative effects of diets lacking in FM and FO. Similarly, other authors [21] fed juvenile flounder with 4.56 and 13.6% *Schizochytrium* and *Nanochloropsis* and obtained similar values to those obtained with FO in terms of body, plasma, and muscle composition. According to these authors, the inclusion of these microalgae as the sole source of lipids was sufficient to obtain good feed efficiency, good growth performance, and healthy values for the consumer. Furthermore, the nutritional efficiency of *Nannochloropsis*, *Chlorella*, and *Schizochytrium* was analysed in juvenile red sea bream, *Pagrus major* [43]. The experimental diets were compared with a control diet without FM as protein, which was replaced by soybean and corn meal. The con-

trol diet did contain FO. The results showed that fish fed *Nannochloropsis* and *Schizochytrium* obtained good growth performance and a considerable fatty acid composition, with no adverse effects observed. In another experiment, four experimental diets were studied in juvenile meagre (*Argyrosomus regius*) for 30 days [44]: a control diet containing 5% FO and 7% rapeseed oil, while in the other three diets, FO was completely replaced by poultry oil alone or by a mixture of poultry oil and one of two commercial algal oils extracted from *Schizochytrium* sp. Their results showed that the mixture of these two microalgal oils could completely replace fish oil in a cost-effective manner in the diets of juvenile sea bass.

According to the results of this study, diets supplemented with algae are not only optimal for the growth and survival (100% in all batches) of *S. aurata* at the end of its grow-out phase of the production cycle but can also be used to reverse the negative effect on growth of diets low or lacking in FM and FO and containing an excessive percentage of terrestrial vegetables. Interestingly, this effect is achieved by means of changes in the muscle cellularity that can affect the fillet quality, mainly the texture, as has been observed by other authors [27,28,30,31]. In general, it is observed that fish with higher values of hyperplasia and density of white muscle fibres show higher values of textural firmness, which is considered a positive parameter for the quality of the fish fillet [27,28]. To study the correlation between the histological data of the present study and the quality of the fillet, our research team is currently performing physicochemical and organoleptic analyses of the fillets of all the groups included in the present study.

5. Conclusions

1. The C2-R diet produced higher body weight values than the other diets. Likewise, the C2-R group showed the highest values of fibrillar hypertrophy.
2. Diets enriched with hydrolysed algae showed a tendency to generate a higher number of fibres in the fish muscles than diets with raw algae biomass, although this was not significant.
3. Diets supplemented with algal biomass (microalgae and macroalgae) were able to reverse the negative effect of a diet low or lacking in FM and FO and rich in vegetable ingredients in terms of feed conversion rate and weight gain.
4. Our data point to algal biomass-containing diets as good alternatives to diets rich in plant ingredients.

Author Contributions: M.D.A., E.C.-P., M.A., M.I.S., F.J.A. and T.F.M. conceived and designed the experiments. Also, all of them carried out the interpretation and discussion of the data; F.J.A., T.F.M. and M.I.S. prepared the aquafeeds. M.A. and E.C.-P. reared the fish. M.A. and E.C.-P. performed the diet trial and the samplings. The body measurements were carried out by M.A. and E.C.-P. The fibrillar analysis and the statistical analysis were carried out by M.D.A. All authors have read and agreed to the published version of the manuscript.

Funding: Funded by MCIN/AEI/10.13039/501100011033 and FEDER (PID2021-122287OB-C21 and C22); and by EU Horizon 2020 (SABANA 727874) which supplied the microalgae biomass.

Institutional Review Board Statement: The authors state that no conflicts, informed consent, or 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 (RD 53/2013, as amended by RD 218/2021), 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 COMU-IEO, CSIC (REGA code ES300261040017—Approval Date 5 April 2016) with the approval of the Ministry of Water, Agriculture and Environment of the Autonomous Community Region of Murcia (Spain; A13230103).

Informed Consent Statement: Not applicable.

Data Availability Statement: Experimental data supporting the findings of this study are available on request.

Acknowledgments: We thank all the staff involved in fish management in the COMU-IEO, CSIC facilities.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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