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# Effects of dietary use of two lipid extracts from the microalga *Nannochloropsis gaditana* (Lubián, 1982) alone and in combination on growth and muscle composition in juvenile gilthead seabream, *Sparus aurata*

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ABSTRACT

Microalgae are a source of diverse high-value compounds, such as carotenoids and fatty acids, which have a potential application in aquafeeds. Some microalgae species present complex cell wall structures, which make them poorly digestible, thus limiting their use as a feed ingredient. Consequently, applying extracted compounds to aquafeeds instead of using the whole algal biomass is advantageous as this is expected to increase the bioavailability of these nutrients. The aim of this study was to evaluate the effect of the dietary inclusion (2%) of two extracts obtained from the microalga *Nannochloropsis gaditana* (one composed of saponifiable lipids and the other of non-saponifiable lipids), alone or in combination, on growth, muscle composition, skin color and lipid oxidation in juvenile gilthead seabream, *Sparus aurata*, following a 39-day trial. Overall, the inclusion of 2% of the saponifiable lipid extract did not affect the growth performance but fish muscle presented a lower percentage of docosahexaenoic acid (DHA) and a higher eicosapentaenoic acid (EPA) than that of the fish fed the diets lacking this lipidic fraction. Despite no effect being observed in fish growth performance, the inclusion of 2% of the non-saponifiable lipid extract enhanced the carotenoid content of the fish muscle, which prevented lipid oxidation, and modulated the skin pigmentation towards a yellow-greenish color. The present study confirms the success of applying both high-value microalgae lipidic extracts, alone or in combination, as feed additives for practical diets in juvenile gilthead seabream.

## 1. Introduction

Currently, great effort has been made on the development of complete aquafeeds that meet the nutritional requirements of aquaculture fish. For this purpose, alternative ingredients for replacing the traditional feedstuffs (fish meal and fish oil) are being evaluated for improving the economic and environmental sustainability of aquaculture [1]. Indeed, the percentage of fish derivatives in the composition of aquafeeds has decreased progressively due to their high price and their diminishing market availability [2]. In this sense, alternatives to fish oil like vegetable oils (such as soy and palm) have been commonly used owing to their low market price. However, they cannot completely substitute fish oil as they do not contain long-chain polyunsaturated fatty acids (LC-PUFAs) such as 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) [3]. Since LC-PUFAs are essential fatty acids and must be provided via the diet [4], the search for alternative sources of LC-PUFA-rich ingredients has increased in the last years.

Biomass obtained from marine microalgae is an alternative LC-PUFA source that can be used to replace fish-derived oil in aquafeeds. It is believed that, within a few years, once microalgae production, harvesting and biomass processing have been improved, the production costs of EPA and DHA from microalgae will be equivalent to that of fish oil [5]. Besides producing LC-PUFAs, microalgae can also synthesize

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other high-value compounds such as carotenoids (β-carotene, astaxanthin), phycobilins, sterols, polyhydroxyalkanoates, polysaccharides and others [6]. In fish, carotenoids act modulating skin and muscle coloration, are precursors of vitamin A, they are antioxidants and immunostimulants, and they affect reproductive performance, besides, they can enhance fish growth performance [7]. Fish cannot produce carotenoids *de-novo* so pigment inclusion must be considered in the feed formulation [8].

Various microalgae species have been tested as feed ingredients for gilthead seabream - *Scenedesmus* sp. [9], *Tetraselmis* sp. [10], *Isochrysis* sp. [11], *Schizochytrium* sp. and *Crypthecodinium cohnii* [12], *Chlorella* sp. [13], *Haematococcus* sp. [14], *Phaeodactylum* sp. [15] and *Nannochloropsis* sp. [16] - as substitutes for fish meal or fish oil that also promoted skin pigmentation and immunostimulation.

The difficulty faced when using microalgae as feed ingredients is that some species have rigid cell walls and might be poorly digestible for some fish, especially carnivorous species. The digestibility of an ingredient is species-specific, *Scenedesmus* sp. and *Tetraselmis* sp. presented lower protein availability compared to *N. gaditana* and *Tisochrysis* sp. by gilthead seabream *S. aurata* and Senegalese sole *Solea senegalensis* when their digestibility was tested using in vitro assays [17]. Substituting fish meal for defatted *Nannochloropsis oceanica* biomass (20%) reduced the digestibility of Atlantic salmon (*Salmo salar*) diets [18]. Scholz et al. [19] stated that *Nannochloropsis* sp. microalgae possess a multilayer cell wall, the outer wall being composed of algaenan, a non-hydrolysable polysaccharide, which makes it difficult to break down. *N. oceanica* presented a mere 35% digestibility of crude protein for mink (*Mustela vison*) [20]. Mechanical and physical processing of *N. gaditana* biomass increased the nutrients accessibility in both in vitro and in vivo tests with Nile tilapia *Oreochromis niloticus* [21]. Other studies suggested that *N. oculata* biomass should be processed before being used to improve protein, lipid, and β-carotene accessibility [22,23]. Still, no study was found using *Nannochloropsis* extracts as feed ingredients.

Recognizing the importance of LC-PUFAs and carotenoids in aquafeeds, and that the nutrients from the microalgae extracts might be more bioaccessible than the biomass as a whole, the objective of this piece of research was to evaluate the effects of feeding diets supplemented with a fatty acid-rich (saponifiable fraction) extract and a carotenoid-rich (non-saponifiable fraction) extract obtained from *N. gaditana*, alone or in combination, on growth performance, nutrient utilization, muscle composition and lipid oxidation and skin color of juvenile gilthead seabream.

## 2. Materials and methods

### 2.1. Production of the microalgae extracts

The microalga *Nannochloropsis gaditana* (Lubián, 1982) was cultured in tubular photobioreactors during the spring-summer of 2018 at the pilot plant (the SABANA facilities) of the University of Almería, Spain as reported by Menegol et al. [24]. The culture pH was maintained at 8 by the on-demand addition of $CO_2$. The culture was harvested daily by centrifugation (at a dilution rate of 0.3 $d^{-1}$) and then the concentrated biomass was freeze-dried.

The biomass was extracted following the procedure described by Sales et al. [25] that allows separating extraction of fatty acids and carotenoids from the microalga *N. gaditana*. In brief, the biomass was saponified using a three-component solution (WIH; water: isopropanol: hexane) and the extract with non-saponified lipids (carotenoid-rich) was separated by partitioning the solution, adjusting the proportion of solvents to separate them. The non-saponifiable lipid extract, present in the hexanic phase, was collected by decantation. The hydroalcoholic phase (with the saponified lipids) was later acidified to pH ~2 to free the fatty acids, which were extracted by adding hexane and water (1:0.6:0.3 - hydroalcoholic extract: water: hexane) and separated by decantation [26]. The extract solvents were later dried in a rotary evaporator.

The composition of the saponifiable lipid extract is shown in Table 1, in terms of fatty acid profile. This extract presented high concentrations of eicosapentaenoic acid (EPA 20:5n-3; 22.1%), palmitic acid (16:0; 23.5%) and palmitoleic acid (16:1n-7; 18.8%); however, it was deficient in docosahexaenoic acid (DHA 22:6n-3). The composition of the non-saponifiable lipid extract is detailed in Table 2, in terms of carotenoid content. Violaxanthin, β-carotene and neoxanthin were the most abundant carotenoids in this extract and vaucheroxanthin ester and cantha-xanthin less abundant.

### 2.2. Production of the experimental diets

Experimental diets were produced at the CEIA3-Universidad de Almería facilities (Experimental Feeds Service; http://www.ual.es/stecnicos_spe) using standard aquafeed procedures, i.e. mixing ingredients, inclusion of feed additives, gentle extrusion with temperature control, and granulation within the size range of experimental feeds. Four experimental diets were formulated to evaluate the inclusion of the microalgal extracts: CT: control diet, with no microalgae extract; D2 with 2% inclusion of the saponifiable lipid (fatty acids) extract; D3: with 2% inclusion of the non-saponifiable lipid (carotenoids) extract; D4: with 2% inclusion of the saponifiable lipid extract and 2% of the non-saponifiable lipid extract.

The ingredients of experimental diets are detailed in Table 3. The dry ingredients were finely ground and mixed in a 10-L Sammic BM-10 vertical helix ribbon mixer (Sammic, Azpeitia, Spain) before adding the fish oil and diluted choline chloride. The microalgae extracts were dispersed in water by emulsion with soybean lecithin before being added to the dry ingredients, and the resulting dough was pelleted into 1 mm pellets using an extruder (model P-100, La Monferrina, Italy). Finally, the pellets were dried in a 12 $m^3$ drying chamber with forced-air circulation (Airfrio, Almería, Spain) at 30 °C for 24 h, then kept in sealed plastic bags at −20 °C until use.

The proximate composition of the experimental diets is detailed in Table 3. The protein, lipid, ash and moisture contents of the diets were similar. The fatty acid profile of the experimental diets is presented in Table 4. In sum, the EPA concentration in D2 and D4 was higher than in diets CT and D3. The DHA contents in the CT, D3 diets were similar and higher than in D2 and D4.

The carotenoid content of the experimental diets is shown in Table 5. The total carotenoid content of the D3 and D4 diets was higher than in the CT, D2. β-Carotene and violaxanthin were the most abundant carotenoids in the D3 and D4 diets; furthermore, their concentration was much higher than in the CT, D2 diets.

**Table 1**
Fatty acid profile (% total fatty acid) of the *N. gaditana* saponifiable lipid extract.

| Fatty acid | Saponifiable lipid extract |
|---|---|
| 14:0 | 7.0 ± 0.0 |
| 16:0 | 23.5 ± 0.4 |
| 16:1n-7 | 18.8 ± 0.3 |
| 16:2n-4 | 0.1 ± 0.0 |
| 16:3n-4 | 0.3 ± 0.0 |
| 18:0 | 0.6 ± 0.0 |
| 18:1n-9 | 6.3 ± 0.2 |
| 18:1n-7 | 0.1 ± 0.0 |
| 18:2n-6 | 2.2 ± 0.0 |
| 18:3n-3 | 0.2 ± 0.0 |
| 18:4n-3 | 0.1 ± 0.0 |
| 20:1n-9 | 0.1 ± 0.0 |
| 20:4n-6 | 0.2 ± 0.1 |
| 20:4n-3 | 5.6 ± 0.3 |
| 20:5n-3 | 22.1 ± 0.1 |
| 22:6n-3 | 0.0 ± 0.0 |
| Other fatty acids | 12.7 ± 1.5 |

Values are mean ± SD of triplicate determination (n = 3).

**Table 2**
Carotenoid content (mg kg$^{-1}$) of the *N. gaditana* non-saponifiable lipid extract.

| Carotenoid | Non-saponifiable lipid extract |
|---|---|
| Neoxanthin | 755.0 ± 149.2 |
| Violaxanthin | 2137.3 ± 255.0 |
| Antheroxanthin | 417.5 ± 70.4 |
| Vaucheroxanthin | 78.8 ± 10.6 |
| Zeaxanthin | 58.3 ± 8.1 |
| Vaucheroxanthin ester | 13.5 ± 2.4 |
| Canthaxanthin | 14.1 ± 2.2 |
| β-Carotene | 925.5 ± 145.4 |
| Total | 4400.0 ± 643.3 |

Values are mean ± SD of triplicate determination (n = 3).

**Table 3**
Ingredient and proximate composition of the experimental diets.*

| | CT | D2 | D3 | D4 |
|---|---|---|---|---|
| Ingredient composition (% dry weight, d.w.) | | | | |
| Fish meal[a] | 15.0 | 15.0 | 15.0 | 15.0 |
| Wheat gluten | 15.0 | 15.0 | 15.0 | 15.0 |
| Soybean meal concentrate | 37.4 | 37.4 | 37.4 | 37.4 |
| Attractant premix[b] | 8.0 | 8.0 | 8.0 | 8.0 |
| Fish oil | 4.0 | 2.0 | 4.0 | 2.0 |
| Soybean oil | 4.0 | 4.0 | 4.0 | 4.0 |
| *N. gaditana* saponifiable lipid extract | 0.0 | 2.0 | 0.0 | 2.0 |
| *N. gaditana* non-saponifiable lipid extract | 0.0 | 0.0 | 2.0 | 2.0 |
| CPSP90[c] | 5.0 | 5.0 | 5.0 | 5.0 |
| Soybean lecithin | 1.0 | 1.0 | 1.0 | 1.0 |
| Lysine | 1.2 | 1.2 | 1.2 | 1.2 |
| Methionine | 0.5 | 0.5 | 0.5 | 0.5 |
| Maltodextrin | 3.8 | 3.8 | 1.8 | 1.8 |
| Choline chloride | 0.5 | 0.5 | 0.5 | 0.5 |
| Betaine | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin and mineral premix[d] | 2.0 | 2.0 | 2.0 | 2.0 |
| Stay C Roche 0.2%[e] | 0.1 | 0.1 | 0.1 | 0.1 |
| Guar gum | 1.0 | 1.0 | 1.0 | 1.0 |
| Alginate | 1.0 | 1.0 | 1.0 | 1.0 |
| Proximate composition (% d.w.) | | | | |
| Crude protein | 55.1 | 55.4 | 55.7 | 55.0 |
| Crude lipid | 13.3 | 15.1 | 15.3 | 16.1 |
| Ash | 6.6 | 6.6 | 7.1 | 7.2 |
| Moisture | 8.2 | 6.6 | 7.8 | 6.1 |

* CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

[a] Protein 69.4%; lipid 12.3%, Norsildemel (Bergen, Norway).
[b] 37.5% krill meal, 62.5% squid meal.
[c] CPSP90; protein: 84.1%; lipid: 8.8%, Sopropeche (France).
[d] Vitamin and mineral premix according to Vizcaíno et al. [9].
[e] Stay C Roche 0.2%.

### 2.3. Experimental design

Juvenile gilthead seabream, *Sparus aurata*, were obtained from a commercial nursery, Predomar S.A. (Almería, Spain). The experimental fish were brought to the Experimental Aquarium at the University of Almería (REGA number ES04013002262) and acclimated for one week before the trial, during which time they were fed a commercial feed (Skretting; feeding rate 5% body weight, BW). After the acclimation, twenty fish (1.5 ± 0.2 g BW) were randomly placed into each tank (volume: 0.04 m$^3$; 0.48 L min$^{-1}$ flow rate) and fed three times a day (9:00, 13:00 and 17:00 h) at 5% BW until they grew to 5 times their initial BW. Each diet was tested in triplicate (4 diets × 3 tanks) for 39 days. The trial was conducted in January–February, the water was artificially salinized, and the tanks communicated with each other by the recirculating water in a RAS system equipped with physical and biological filters and a protein skimmer. The temperature was maintained at 21 ± 0.6 °C, salinity at 30 ± 1.5 PSU, ammonia below 1 mg L$^{-1}$ and a photoperiod of 12 L:12D. All experimental procedures complied with the European Union Guidelines (Directive 2010/63/UE) for the use of laboratory animals. The Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 06/02/2020/011).

At the beginning, all the fish were anesthetized by clove oil overdose (30 ppm), and individually weighed. During the experiment, fish in each tank were individually weighed at 15 and 39 days, after fasting for 24 h. Standard length was measured at the end of the experiment.

At day 39, the fish were sacrificed using a clove oil overdose (200 ppm), after which their spines were severed. The digestive tract and liver were excised from all fish. The skin color parameters were measured immediately from a pool of five fish from each tank. A pool of five fish from each tank was frozen to determine the muscle lipid oxidation. The remain carcasses and the livers were freeze-dried and stored at −20 °C to analyze the muscle proximate composition, the fatty acid profiles from

**Table 4**
Fatty acid profile (% total fatty acid) of the experimental diets.

| Fatty acid | CT | D2 | D3 | D4 |
|---|---|---|---|---|
| 14:0 | 1.7 ± 0.0 | 2.7 ± 0.0 | 2.2 ± 0.0 | 2.8 ± 0.1 |
| 16:0 | 15.3 ± 0.0 | 17.1 ± 0.1 | 16.1 ± 0.0 | 17.3 ± 0.2 |
| 16:1n-7 | 2.3 ± 0.0 | 5.5 ± 0.0 | 3.9 ± 0.0 | 6.2 ± 0.2 |
| 16:2n-4 | 0.6 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.0 |
| 16:3n-4 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 |
| 18:0 | 4.7 ± 0.0 | 3.8 ± 0.0 | 4.3 ± 0.0 | 3.6 ± 0.0 |
| 18:1n-9 | 17.7 ± 0.0 | 16.4 ± 0.1 | 16.6 ± 0.0 | 15.8 ± 0.2 |
| 18:1n-7 | 2.1 ± 0.0 | 1.7 ± 0.0 | 1.9 ± 0.0 | 1.6 ± 0.0 |
| 18:2n-6 | 26.7 ± 0.1 | 26.1 ± 0.1 | 24.6 ± 0.0 | 24.8 ± 0.2 |
| 18:3n-3 | 3.2 ± 0.0 | 3.0 ± 0.0 | 2.9 ± 0.0 | 2.9 ± 0.0 |
| 18:4n-3 | 0.7 ± 0.0 | 0.5 ± 0.0 | 0.6 ± 0.0 | 0.5 ± 0.0 |
| 20:1n-9 | 2.3 ± 0.0 | 1.9 ± 0.0 | 2.1 ± 0.0 | 1.8 ± 0.0 |
| 20:4n-6 | 0.8 ± 0.0 | 1.3 ± 0.0 | 1.2 ± 0.0 | 1.6 ± 0.0 |
| 20:4n-3 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 |
| 20:5n-3 | 4.5 ± 0.0 | 6.8 ± 0.1 | 6.3 ± 0.0 | 7.9 ± 0.1 |
| 22:5n-3 | 0.9 ± 0.0 | 0.6 ± 0.0 | 0.8 ± 0.0 | 0.6 ± 0.0 |
| 22:6n-3 | 10.8 ± 0.1 | 7.0 ± 0.2 | 9.9 ± 0.0 | 6.7 ± 0.1 |
| Other FA | 5.4 ± 0.1 | 4.6 ± 0.0 | 5.4 ± 0.1 | 5.2 ± 0.1 |
| SFA | 21.7 ± 0.0 | 23.6 ± 0.1 | 22.6 ± 0.1 | 23.7 ± 0.3 |
| MUFA | 24.4 ± 0.0 | 25.5 ± 0.1 | 24.6 ± 0.0 | 25.4 ± 0.0 |
| LC-PUFA | 17.2 ± 0.1 | 16.0 ± 0.3 | 18.5 ± 0.1 | 16.9 ± 0.1 |
| n-3 | 20.2 ± 0.1 | 18.2 ± 0.3 | 20.8 ± 0.1 | 18.7 ± 0.1 |
| n-6 | 27.5 ± 0.1 | 27.4 ± 0.1 | 25.8 ± 0.0 | 26.4 ± 0.2 |
| n-9 | 20.0 ± 0.0 | 18.3 ± 0.1 | 18.7 ± 0.0 | 17.6 ± 0.2 |
| n-3/n-6 | 0.7 ± 0.0 | 0.7 ± 0.0 | 0.8 ± 0.0 | 0.7 ± 0.0 |
| EPA/DHA | 0.4 ± 0.0 | 1.0 ± 0.0 | 0.6 ± 0.0 | 1.2 ± 0.0 |

Values are mean ± SD of triplicate determination (n = 3). FA: fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

**Table 5**
Carotenoid content (mg kg$^{-1}$) of the experimental diets.

| Carotenoid | CT | D2 | D3 | D4 |
|---|---|---|---|---|
| Neoxanthin | 0.2 ± 0.1 | 3.8 ± 4.3 | 66.8 ± 1.0 | 82.0 ± 2.2 |
| Violaxanthin | 0.2 ± 0.0 | 2.5 ± 0.4 | 257.6 ± 10.7 | 221.4 ± 12.4 |
| Antheroxanthin | | 1.6 ± 0.2 | 40.4 ± 0.8 | 32.8 ± 2.3 |
| Vaucheroxanthin | | 0.8 ± 1.1 | 8.2 ± 0.3 | 8.8 ± 0.5 |
| Zeaxanthin | 0.8 ± 0.0 | 3.0 ± 0.1 | 9.4 ± 0.5 | 12.5 ± 0.6 |
| Vaucheroxanthin ester | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.6 ± 0.6 | 0.3 ± 0.1 |
| Canthaxanthin | | 0.1 ± 1.7 | 2.0 ± 9.4 | 2.5 ± 12.9 |
| β-Carotene | 0.2 ± 0.1 | 9.4 ± 1.7 | 277.6 ± 9.4 | 297.1 ± 12.9 |
| Total | 1.6 ± 0.1 | 21.5 ± 0.4 | 662.7 ± 21.8 | 657.3 ± 30.5 |

Values are mean ± SD of triplicate determination (n = 3). *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

muscle and liver, and carotenoid content of the muscle.

### 2.4. Growth performance

The following growth parameters were evaluated: the specific growth rate (SGR, %) = (Ln ($W_f$) − Ln ($W_i$)/days) × 100, where $W_f$ and $W_i$ were the final and the initial weight (g); the daily gain (DG, g $day^{-1}$) = ($W_f$ − $W_i$)/days; the feed conversion ratio (FCR) = total feed intake (g)/weight gain (g); and the condition factor (k) = $W_f/L_f^3$, where L is the final length.

Biometric indices were estimated in accordance with the following equations: the Viscerosomatic Index (VI, %) = [visceral weight (g)/body weight (g)] × 100; and the Hepatosomatic Index (HSI, %) = [liver weight (g)/body weight (g)] × 100; Intestinal Quotient (IQ) = $L_i/L_b$, where $L_i$ and the $L_b$ are the intestine and body length.

### 2.5. Analytical procedures

The chemical analysis (dry matter and ash) of the aquafeeds and fish muscle was performed following the Association of Official Agricultural Chemists AOAC [27] methods. The crude protein (N × 6.25) was determined by elemental analysis (C: H: N) using a Fisons EA 1108 analyzer (Fisons Instruments, USA). The total lipid content was analyzed in accordance with Folch et al. [28] using chloroform/methanol (2:1 v/v) as solvent, and total lipid content was calculated gravimetrically.

The carotenoid concentration in aquafeeds, muscle and microalgae extracts was measured by liquid chromatography [29]. The fatty acid profile in the feeds, muscle and liver was measured by gas chromatography [30].

Lipid oxidation was estimated by thiobarbituric acid-reactive substances (TBARS) analysis in fresh muscle according to the method of Buege and Aust [31]. Samples (1 g each) were homogenized in 2 mL of 50 mM $NaH_2PO_4$, 0.1% (v/v) Triton X-100 solution. The mixture was centrifuged (10,000*g*, 20 min, 4 °C) and the supernatants were mixed in a 1:5 (v/v) ratio with 2-thiobarbituric acid (TBA) reagent (0.375% w/v TBA, 15% w/v TCA, 0.01% w/v 2,6-dibutyl hydroxytoluene (BHT) and 0.25 N HCl). The mixture was heated for 15 min, then centrifuged (3600*g*, 10 min, 4 °C); the supernatant's absorbance was measured at 535 nm. The amount of TBARS was expressed as mg of malonyl dialdehyde (MDA) per kg of muscle after comparing with a MDA standard.

Instrumental color was measured on a portion of dorsal skin by the $L^*$, $a^*$ and $b^*$ system [32] using a Minolta Chroma meter CR400 device (Minolta, Osaka, Japan). The parameters for lightness ($L^*$; on a 0–100 point scale from black to white), redness ($a^*$; assessing the position between red positive values and green negative values), and yellowness ($b^*$; assessing the position between yellow positive values and blue negative values) were recorded.

### 2.6. Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analysis was performed in Statistica 7 software (Statsoft, USA). Data on growth performance, biometric indices, proximate composition of muscle, lipid oxidation and skin color were checked for homogeneity and normality. The data (in percentage) was arcsine square root transformed. Where necessary, the data was log(x + 1) transformed to meet the parametric requirements and compared by one-way analysis of variance (ANOVA); significantly different treatments ($p < 0.05$) were determined by Tukey's post-hoc test. Data with a nonparametric distribution were analyzed using the Kruskal-Wallis test.

## 3. Results

### 3.1. Growth performance

Growth performance and biometric indexes are shown in Table 6.

**Table 6**
Growth performance, nutrient utilization and biometric indexes (mean ± SD) of juvenile gilthead seabream, *Sparus aurata*, at day 39 of the feeding trial. Superscript letters indicate significant difference between experimental diets ($p < 0.05$).

| | CT | D2 | D3 | D4 | *p*-Value |
|---|---|---|---|---|---|
| Growth and nutrient utilization | | | | | |
| Initial body weight (g) | 1.6 ± 0.3 | 1.5 ± 0.2 | 1.5 ± 0.3 | 1.5 ± 0.2 | 0.247 |
| Final body weight (g) | 7.5 ± 0.8 | 7.1 ± 1.2 | 7.3 ± 1.1 | 7.6 ± 1.2 | 0.324 |
| Length (cm) | 8.0 ± 0.4 | 7.8 ± 0.5 | 7.8 ± 0.3 | 7.9 ± 0.4 | 0.183 |
| Daily gain (g $day^{-1}$) | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.205 |
| Specific growth rate | 4.0 ± 0.2 | 4.0 ± 0.4 | 4.0 ± 0.3 | 4.3 ± 0.3 | 0.056 |
| Feed conversion rate | 1.9 ± 0.3 | 1.9 ± 0.3 | 1.7 ± 0.3 | 1.8 ± 0.3 | 0.054 |
| k | 14.6 ± 1.2 | 15.0 ± 1.0 | 15.3 ± 1.0 | 15.3 ± 0.9 | 0.175 |
| Somatic indexes | | | | | |
| VI | 5.1 ± $0.8^{b}$ | 5.6 ± $0.9^{ab}$ | 5.6 ± $0.8^{ab}$ | 6.1 ± $0.7^{a}$ | 0.005 |
| HSI | 1.5 ± 0.3 | 1.6 ± 0.3 | 1.6 ± 0.3 | 1.5 ± 0.3 | 0.121 |
| IQ | 0.7 ± 0.1 | 0.8 ± 0.1 | 0.7 ± 0.1 | 0.8 ± 0.1 | 0.094 |

Values are mean ± SD of triplicate tanks. k: condition factor, VI: viscerosomatic index, HSI: hepatosomatic index, IQ: intestinal quotient. *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

Overall, fish multiplied their initial body weight by 4.9-fold at the end of the feeding trial. No significant differences on growth performance and nutrient utilization were observed among the dietary treatments. The VI in D4-fed fish was higher than in fish fed the CT diet while the HSI remained unaffected. Fish accepted all the experimental feeds, and mortality was low throughout the experiment (<5%).

### 3.2. Muscle chemical composition and fatty acid profile

Chemical composition of muscle was not affected by the dietary treatments (Table 7). The fatty acid profile of liver is shown in Table 8. Feeding the fish the diets supplemented with microalgae extracts significantly increased EPA and decreased DHA percentages compared to CT group. However, the total LC-PUFA and total n-3 fatty acids were similar in all the dietary treatments. In addition, fish fed the CT diet showed the highest and the lowest n-3/n-6 and EPA/DHA ratios, respectively.

The muscle fatty acid profiles are shown in Table 9. The EPA content in fish fed the D4 diet was significantly higher than in fish fed the other diets. CT and D3 groups showed the highest DHA content, and the percentage of SFA was similar in all treatments. The percentage of MUFA in the muscle of CT fish was significantly higher than in fish fed

**Table 7**
Muscle chemical composition* (% d.w., mean ± SD) of juvenile gilthead seabream, *Sparus aurata*, at the end of the feeding trial. Letters indicate significant difference between experimental diets ($p < 0.05$).

| | CT | D2 | D3 | D4 | p-Value |
|---|---|---|---|---|---|
| Protein | 73.1 ± 3.7 | 70.8 ± 2.4 | 73.4 ± 2.0 | 70.5 ± 2.5 | 0.326 |
| Lipid | 14.5 ± 0.9 | 16.7 ± 0.5 | 15.8 ± 1.1 | 15.7 ± 1.1 | 0.128 |
| Ash | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.6 ± 0.1 | 1.5 ± 0.2 | 0.485 |
| Moisture | 77.1 ± 1.0 | 78.4 ± 0.5 | 77.6 ± 1.1 | 77.1 ± 0.1 | 0.943 |

Values are mean ± SD of triplicate determination ($n = 3$). *Percentage of biomass dry weight (d.w.). *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

**Table 8**
Liver fatty acid profile* (% total fatty acid) in juvenile gilthead seabream, *Sparus aurata*, at the end of the feeding trial. Superscript letters indicate significant difference between experimental diets ($p < 0.05$).

| Fatty acid | CT | D2 | D3 | D4 | *p*-Value |
|---|---|---|---|---|---|
| 14:0 | 1.4 ± 0.0$^{b}$ | 1.7 ± 0.03$^{a}$ | 1.6 ± 0.0$^{a}$ | 1.7 ± 0.0$^{a}$ | <0.0001 |
| 16:0 | 16.7 ± 0.2 | 17.1 ± 0.5 | 16.2 ± 0.0 | 16.7 ± 0.0 | 0.0303 |
| 16:1n-7 | 2.6 ± 0.0$^{d}$ | 4.2 ± 0.1$^{b}$ | 3.6 ± 0.0$^{c}$ | 4.6 ± 0.1$^{a}$ | <0.0001 |
| 16:2n-4 | 0.8 ± 0.1 | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.0187 |
| 16:3n-4 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.0 | 0.4 ± 0.0 | 0.0668 |
| 18:0 | 7.8 ± 0.1$^{ab}$ | 7.9 ± 0.4$^{a}$ | 7.3 ± 0.0$^{b}$ | 8.1 ± 0.1$^{a}$ | 0.0008 |
| 18:1n-9 | 25.2 ± 0.5 | 26.1 ± 1.4 | 24.8 ± 0.0 | 25.5 ± 0.1 | 0.0592 |
| 18:1n-7 | 2.6 ± 0.1$^{c}$ | 3.0 ± 0.1$^{b}$ | 2.9 ± 0.0$^{b}$ | 3.2 ± 0.0$^{a}$ | <0.0001 |
| 18:2n-6 | 16.0 ± 0.4 | 16.0 ± 1.3 | 16.9 ± 0.1 | 16.0 ± 0.0 | 0.2327 |
| 18:3n-3 | 1.5 ± 0.1 | 1.4 ± 0.1 | 1.6 ± 0.0 | 1.4 ± 0.0 | 0.0592 |
| 18:4n-3 | 0.5 ± 0.0$^{a}$ | 0.6 ± 0.0$^{a}$ | 0.7 ± 0.0$^{b}$ | 0.5 ± 0.0$^{a}$ | <0.0001 |
| 20:1n-9 | 1.1 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 0.0475 |
| 20:4n-6 | 1.4 ± 0.0$^{c}$ | 1.6 ± 0.1$^{b}$ | 1.6 ± 0.0$^{b}$ | 1.8 ± 0.0$^{a}$ | <0.0001 |
| 20:4n-3 | 0.3 ± 0.0$^{a}$ | 0.2 ± 0.0$^{b}$ | 0.3 ± 0.0$^{b}$ | 0.2 ± 0.0$^{b}$ | <0.0001 |
| 20:5n-3 | 3.0 ± 0.0$^{b}$ | 3.7 ± 0.3$^{a}$ | 3.7 ± 0.0$^{a}$ | 4.0 ± 0.1$^{a}$ | <0.0001 |
| 22:5n-3 | 0.8 ± 0.1 | 0.8 ± 0.0 | 0.8 ± 0.0 | 0.9 ± 0.0 | 0.0598 |
| 22:6n-3 | 10.8 ± 0.1$^{a}$ | 7.2 ± 0.6$^{c}$ | 9.0 ± 0.1$^{b}$ | 6.6 ± 0.1$^{c}$ | <0.0001 |
| Other FA | 7.4 ± 0.4 | 7.0 ± 0.4 | 7.3 ± 0.1 | 7.2 ± 0.2 | 0.3703 |
| Total FA* | 15.8 ± 1.0 | 15.4 ± 1.7 | 18.2 ± 1.2 | 17.8 ± 0.2 | 0.0603 |
| SFA | 25.9 ± 0.3 | 26.8 ± 0.9 | 25.1 ± 0.0 | 26.5 ± 0.0 | 0.0687 |
| MUFA | 31.5 ± 0.6 | 34.3 ± 1.4 | 32.1 ± 0.3 | 34.3 ± 0.0 | 0.0493 |
| LC-PUFA | 16.3 ± 0.1 | 13.5 ± 1.0 | 15.4 ± 0.2 | 13.5 ± 0.3 | 0.0587 |
| n-3 | 16.9 ± 0.2 | 14.0 ± 1.1 | 16.0 ± 0.1 | 13.7 ± 0.2 | 0.2263 |
| n-6 | 17.5 ± 0.4 | 17.5 ± 1.4 | 18.5 ± 0.1 | 17.8 ± 0.0 | 0.0756 |
| n-9 | 26.3 ± 0.5 | 27.1 ± 1.4 | 25.7 ± 0.3 | 26.5 ± 0.1 | 0.4544 |
| n-3/n-6 | 1.0 ± 0.0$^{a}$ | 0.8 ± 0.0$^{c}$ | 0.8 ± 0.0$^{b}$ | 0.8 ± 0.0$^{c}$ | <0.0001 |
| EPA/DHA | 0.3 ± 0.0$^{d}$ | 0.5 ± 0.0$^{b}$ | 0.4 ± 0.0$^{c}$ | 0.6 ± 0.0$^{a}$ | <0.0001 |

Values are mean ± SD of triplicate determination ($n = 3$). *Percentage of biomass dry weight. FA: fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

the diets D2, D3 and D4. The highest percentage of LC-PUFA was found in fish fed the D3 diet, while CT, D2 and D4-fed fish showed intermediate values. Fish fed the CT diet showed the lowest EPA/DHA ratio.

### 3.3. Muscle biochemical composition – carotenoids

The carotenoid content in fish muscle is shown in Table 10. Fish fed the D3 and D4 diets presented significantly higher total carotenoid content in comparison to specimens fed the CT and D2 diets. The neoxanthin and violaxanthin concentrations were similar in all dietary groups. D3 and D4-fed fish presented similar concentration of zeaxanthin, and higher than fish fed the other diets. The highest muscle β-carotene content was found in fish fed the D3 and D4 diets while the ones fed the CT and D2 treatments presented the lowest.

### 3.4. Skin color and muscle lipid oxidation

The fish skin color parameters are detailed in Table 11. L* was not affected by the diets whereas a* values were higher in the skin of fish fed the D3 and D4 diets than in those fed CT and D2. The skin yellowness (b*) in D2, D3 and D4 groups was significantly higher than in CT fish. The muscle lipid oxidation values (measured as TBARS content) are shown in Fig. 1. Fish fed the CT diet presented the highest TBARS content in the muscle. The non-saponifiable lipid (carotenoid fraction) extract included in the D3 and D4 diets reduced muscle lipid oxidation, which was significantly lower than in fish fed the CT and D2 diets.

**Table 9**
Muscle fatty acid profile* (% total fatty acid) in juvenile gilthead seabream, *Sparus aurata*, at the end of the feeding trial. Superscript letters indicate significant difference between experimental diets ($p < 0.05$).

| Fatty acid | CT | D2 | D3 | D4 | p-Value |
|---|---|---|---|---|---|
| 14:0 | 1.8 ± 0.0 | 2.0 ± 0.0 | 2.3 ± 0.6 | 2.1 ± 0.1 | 0.0761 |
| 16:0 | 17.6 ± 0.1 | 17.0 ± 0.1 | 16.0 ± 1.5 | 16.3 ± 0.2 | 0.0631 |
| 16:1n-7 | 3.9 ± 0.3$^{c}$ | 5.9 ± 0.0$^{a}$ | 4.9 ± 0.1$^{b}$ | 6.3 ± 0.1$^{a}$ | <0.0001 |
| 16:2n-4 | 0.3 ± 0.0$^{ab}$ | 0.3 ± 0.0$^{b}$ | 0.4 ± 0.1$^{a}$ | 0.2 ± 0.0$^{b}$ | 0.0003 |
| 16:3n-4 | 0.4 ± 0.0 | 0.3 ± 0.0 | 0.4 ± 0.1 | 0.3 ± 0.0 | 0.0651 |
| 18:0 | 5.2 ± 0.1 | 4.7 ± 0.0 | 4.5 ± 0.6 | 4.6 ± 0.0 | 0.0636 |
| 18:1n-9 | 25.1 0.1$^{a}$ | 22.3 ± 0.3$^{b}$ | 22.4 ± 0.7$^{b}$ | 21.2 ± 0.1$^{c}$ | <0.0001 |
| 18:1n-7 | 2.6 ± 0.0 | 2.6 ± 0.0 | 2.9 ± 0.4 | 2.6 ± 0.0 | 0.0445 |
| 18:2n-6 | 19.7 ± 0.5 | 21.0 ± 0.4 | 20.1 ± 1.1 | 21.1 ± 0.1 | 0.0894 |
| 18:3n-3 | 2.1 ± 0.1 | 2.2 ± 0.0 | 2.5 ± 0.3 | 2.2 ± 0.0 | 0.0856 |
| 18:4n-3 | 0.5 ± 0.0 | 0.5 ± 0.0 | 0.7 ± 0.3 | 0.5 ± 0.0 | 0.1451 |
| 20:1n-9 | 1.4 ± 0.1 | 1.3 ± 0.0 | 1.4 ± 0.1 | 1.3 ± 0.0 | 0.0894 |
| 20:4n-6 | 0.7 ± 0.0 | 1.2 ± 0.0 | 1.0 ± 0.2 | 1.4 ± 0.0 | 0.05190 |
| 20:4n-3 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.4 ± 0.2 | 0.3 ± 0.0 | 0.04891 |
| 20:5n-3 | 2.9 ± 0.1$^{c}$ | 4.6 ± 0.1$^{b}$ | 4.4 ± 0.2$^{b}$ | 5.6 ± 0.1$^{a}$ | <0.0001 |
| 22:5n-3 | 0.8 ± 0.0 | 0.9 ± 0.1 | 1.6 ± 0.7 | 1.0 ± 0.0 | 0.0743 |
| 22:6n-3 | 9.1 ± 0.1$^{a}$ | 7.7 ± 0.01$^{b}$ | 9.5 ± 0.0$^{a}$ | 7.4 ± 0.1$^{b}$ | <0.0001 |
| Other FA | 5.5 ± 0.4 | 5.0 ± 0.9 | 4.6 ± 1.6 | 5.5 ± 0.3 | 0.4768 |
| Total FA* | 14.5 ± 0.1$^{a}$ | 11.5 ± 0.2$^{b}$ | 13.5 ± 1.3$^{a}$ | 13.0 ± 0.3$^{ab}$ | <0.0001 |
| SFA | 24.6 ± 0.2 | 23.8 ± 0.1 | 22.8 ± 1.5 | 23.0 ± 0.3 | 0.0543 |
| MUFA | 33.0 ± 0.3$^{a}$ | 32.2 ± 0.3$^{ab}$ | 31.5 ± 0.6$^{b}$ | 31.4 ± 0.1$^{b}$ | 0.0002 |
| LC-PUFA | 13.8 ± 0.1$^{c}$ | 14.7 ± 0.3$^{bc}$ | 16.9 ± 0.9$^{a}$ | 15.6 ± 0.2$^{b}$ | <0.0001 |
| n-3 | 15.8 ± 0.1 | 16.1 ± 0.3 | 19.2 ± 1.7 | 16.9 ± 0.2 | 0.0917 |
| n-6 | 20.5 ± 0.5$^{b}$ | 22.2 ± 0.4$^{a}$ | 21.1 ± 1.3$^{ab}$ | 22.6 ± 0.2$^{a}$ | 0.0001 |
| n-9 | 26.5 ± 0.1$^{a}$ | 23.6 ± 0.3$^{b}$ | 23.8 ± 0.8$^{b}$ | 22.5 ± 0.1$^{b}$ | 0.0001 |
| n-3/n-6 | 0.8 ± 0.0 | 0.7 ± 0.0 | 0.9 ± 0.1 | 0.7 ± 0.0 | 0.0491 |
| EPA/DHA | 0.3 ± 0.0$^{d}$ | 0.6 ± 0.0$^{b}$ | 0.5 ± 0.0$^{c}$ | 0.7 ± 0.0$^{a}$ | <0.0001 |

Values are mean ± SD of triplicate determination (n = 3). *Percentage of biomass dry weight. FA: fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

## 4. Discussion

The results obtained in the present work revealed that 2% inclusion of the saponifiable lipid (fatty acid fraction) extract, the non-saponifiable (carotenoid fraction) extract, and the combination of both extracts obtained from *N. gaditana* had no negative effect on growth performance, the nutrient utilization parameters and the somatic indexes in gilthead seabream juveniles. The inclusion of the saponifiable lipid extract was performed by replacing 50% of fish oil. The composition of the microalgae saponifiable lipid extract differed from the fish oil owing to it lacked DHA and contained higher amount of EPA. Therefore, the inclusion of the saponifiable lipid extract altered the fatty acid profile in the D2 and D4 diets, which was expected to influence the performance of the fish. Similar results have been observed in previous works where partial replacement of fish oil (40%) for *Schizochytrium limacinum* meal, rich in DHA, had no detrimental effects on growth of the giant grouper, *Epinephelus lanceolatus* [33]. Similarly, the growth of Atlantic salmon parr was not affected by substituting fish oil (partially or fully) with thraustochytrid oil extract from *Schizochytrium* sp. [34].

Several authors have investigated the fatty acid requirements of juvenile *S. aurata*, and the effect of feeding the fish with low essential fatty acid-diets on growth performance and metabolism [4,35–39]. In this regard, diets for juvenile gilthead seabream must include a minimum of

**Table 10**
Carotenoid content (mg $kg^{-1}$) in the muscle of juvenile gilthead seabream, *Sparus aurata*, at the end of the feeding trial. Superscript letters indicate significant difference between experimental diets ($p < 0.05$).

| Carotenoid | CT | D2 | D3 | D4 | p-Value |
|---|---|---|---|---|---|
| Neoxanthin | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.4201 |
| Violaxanthin | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.0 | 0.3 ± 0.0 | 0.1297 |
| Antheroxanthin | ND | ND | ND | ND | |
| Vaucheroxanthin | ND | ND | ND | ND | |
| Zeaxanthin | 0.2 ± $0.0^{b}$ | 0.5 ± $0.1^{ab}$ | 0.8 $0.2^{a}$ | 1.0 ± $0.0^{a}$ | 0.0034 |
| Vaucheroxanthin ester | ND | ND | ND | ND | |
| Canthaxanthin | ND | ND | ND | ND | |
| β-Carotene | 0.3 ± $0.2^{b}$ | 0.3 ± $0.1^{b}$ | 1.1 ± $0.0^{a}$ | 1.2 ± $0.2^{a}$ | 0.0080 |
| Total | 1.0 ± $0.3^{c}$ | 1.3 ± $0.3^{b}$ | 2.5 ± $0.2^{ab}$ | 2.6 ± $0.2^{a}$ | 0.0129 |

Values are mean ± SD of triplicate determination (n = 3). ND: not detected. *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

**Table 11**
Skin color parameters (L*, a*, b* according to CIE (1986)) in juvenile gilthead seabream, *Sparus aurata*, at the end of the feeding trial. Superscript letters indicate significant difference between experimental diets ($p < 0.05$). Values are mean ± SD of triplicate determination (n = 3).

| Parameter | CT | D2 | D3 | D4 | p-Value |
|---|---|---|---|---|---|
| L* | 58.7 ± 5.6 | 56.4 ± 6.8 | 59.5 ± 4.6 | 59.6 ± 3.9 | 0.304 |
| a* | $-0.5 \pm 0.1^{b}$ | $-0.5 \pm 0.1^{b}$ | $-2.3 \pm 0.5^{a}$ | $-3.0 \pm 0.7^{a}$ | <0.001 |
| b* | $3.9 \pm 0.6^{b}$ | $7.1 \pm 0.9^{a}$ | $7.9 \pm 1.0^{a}$ | $7.8 \pm 1.3^{a}$ | <0.001 |

Color parameters *L**, *a** and *b** stand for lightness, redness, and yellowness, respectively. *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids).

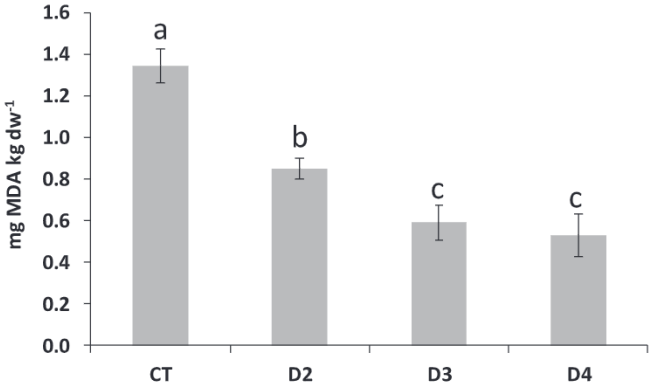


**Fig. 1.** Muscle lipid oxidation of juvenile gilthead seabream, *Sparus aurata*, at the end of the feeding trial. Letters indicate significant difference between experimental diets ($p < 0.05$). Values are mean ± SD of triplicate determination ($n = 3$). *CT (control diet, without microalgae extract); D2 (2% inclusion of extract with saponifiable lipids); D3 (2% inclusion of the extract with non-saponifiable lipids); D4 (2% inclusion of extract with saponifiable lipids and 2% inclusion of the extract with non-saponifiable lipids). MDA: malonyl dialdehyde. d.w.: dry weight.

0.7 and 0.6% d.w. of EPA and DHA, respectively [35]. Moreover, it has been observed that a dietary EPA/DHA ratio of 2:1 improved the growth of *S. aurata* compared to those fish fed diets with ratios of 1:1 and 1:2 [37]. In comparison to the above studies, the composition of the diets that partially substituted the fish oil with the microalgae saponifiable lipid extract (D2 and D4) fulfilled the minimum requirement of essential fatty acids. The content of DHA and EPA (expressed in g/100 g d.w.) can be calculated considering the total fatty acid content of 9.6 and 11.0 in the diets D2 and D4, respectively, and the lipid content in those diets. That way, D2 and D4 contained 0.7 and 0.9 g/100 g d.w. of EPA, respectively, and 0.7 g/100 g d.w. of DHA in both diets. The EPA/DHA ratios in diets D2 and D4 were 1.0 and 1.2, respectively. Consequently, the growth performance was not affected by the partial substitution of fish oil with the microalgal extract.

Related to muscle composition, it was observed that the dietary supplementation with microalgae extract had no effects on the protein and total lipid content. On the other hand, the dietary inclusion of these microalgae extracts had a noticeable influence on muscle and liver fatty acid profiles. Hence, muscle from fish fed the D2 and D4 diets presented a lower DHA concentration, and higher EPA, than those fish fed the CT and D3 diets. Similarly, the muscle fatty acid profile (18:3n-3 and 18:4n-3) of juvenile European sea bass *Dicentrarchus labrax* was altered when fed a diet with 20% inclusion of *Tetraselmis suecica* biomass [40]. On the other hand, no alteration was observed in either growth or muscle fatty acid profile of juvenile gilthead seabream fed diets with different DHA and EPA contents [38]. Despite presenting significantly smaller percentage of DHA in the muscle, the difference between the treatments CT, D2, D3 and D4 treatments was quite small. Even so, the composition of the saponifiable lipid extract could be improved to meet fish requirements by using more than one microalga species, which have complementary fatty acid profiles - one option would be to use the microalgae *Tisochrysis* sp. with *N. gaditana*. In that way, the inclusion of *Tisochrysis* sp. meal did not adversely affect the feed intake of European sea bass, *Dicentrarchus labrax* [41].

Carotenoids are used in feeds for salmonids, shrimp, lobsters and seabream to achieve the required muscle color and to stimulate immune system [42]. However there is still no consensus about the growth stimulation promoted by carotenoids since some studies have reported a positive effect while others found no effect at all [7]. The application of synthetic carotenoids as food dyes is decreasing due to certain health concerns, such as evidence that they cause carcinogenesis and toxicity in the liver and kidneys [43]; for this reason, microalgae have been studied as a natural source of carotenoids. In the present work, no benefit was observed on growth performance and the somatic indexes of the fish fed diets with the inclusion of the non-saponifiable lipid extract. A similar result was observed by Gouveia et al. [13], who reported no differences in the body weight, specific growth rate or feed efficiency ratio of gilthead seabream fed diets with carotenoids included.

Regarding retention of carotenoids, it is widely known that is species-dependent, i.e. each group of organisms presents a particular mechanism for absorbing, metabolizing and depositing carotenoids [7,11]. The level of carotenoids in fish muscle can reach 26–39 mg $kg^{-1}$ in sockeye salmon, *Oncorhynchus nerka*, 8–9 mg $kg^{-1}$ in chinook salmon *Oncorhynchus tshawytscha*, and >3 mg $kg^{-1}$ in rainbow trout, *O. mykiss* [8,12]; such values are much higher than those obtained in the present study. In this work, the accumulation of carotenoids in the muscle of fish fed the carotenoid extract-supplemented diets (diets D3 and D4) was significantly higher than in the muscle of those fish fed the CT and D2 diets. The total carotenoid content of the D3 and D4 diets was similar than those observed in diets tested for barramundi, *L. calcarifer* (650 mg $kg^{-1}$ of phycocyanin, 65 mg $kg^{-1}$ of chlorophyll and 500 mg $kg^{-1}$ of astaxanthin) [44], and higher than that in the diets used for flesh pigmentation of farmed rainbow trout, *Oncorhynchus mykiss*, or Pacific salmon, *Oncorhynchus* spp. (50–100 mg pigment $kg^{-1}$ feed) [8], and even in other diets for gilthead seabream (27–43 mg pigment $kg^{-1}$ feed) [13]. Although there were plenty of carotenoids in the feed, their

deposition in the muscle was lower than expected - 2.5 and 2.6 mg $kg^{-1}$ for diets D3 and D4, respectively - while the control presented 1.0 mg $kg^{-1}$ in the muscle which might have been obtained through the commercial feed provided during the acclimation period before starting the feeding trial. As with our results, low carotenoid deposition, of less than 1 mg $kg^{-1}$, was observed in the muscle of gilthead seabream fed diets including *Chlorella vulgaris* biomass and synthetic astaxanthin, with most of the carotenoids deposited in the skin [13]. These effects could be related with the dietary lipid content and the feeding time which may affect the carotenoid retention in the muscle [45]. In this regards, feeding *S. aurata* a higher lipid content diet, or over a longer period, could have increased carotenoid retention.

Even though it was not measured, we hypothesize that carotenoids might have been deposited in the skin instead of in the muscle. Evidence supporting carotenoid deposition in the skin was the altered skin color parameters of the fish fed diets that included the non-saponifiable lipid extracts (D3 and D4) - the a* values indicated a greater green color in the skin, resulting from the chlorophylls in the microalgae extract; the increase in b* values resulted in greater skin yellowness, related to the high content of yellow pigments, such as β-carotene and zeaxanthin, in the diets and muscle. Tibaldi et al. [41] observed, as we did in the present study, that the lightness of European sea bass skin was not affected by the inclusion of *Isochrysis* sp. biomass whereas the redness and yellowness were. The inclusion of an extract, or the whole biomass, of *Haematococcus pluvialis* slightly increased the carotenoid content in the muscle of *Paralichthys olivaceus* compared to the skin content, the latter presenting a reddish color caused by astaxanthin deposition from that microalgae [46]. A practical application of the visual appearance of fish fed the microalgae extracts is the easy differentiation of batches of fish fed a premium diet, with functional nutrients, from those fed regular diets. Another benefit of including carotenoids in aquafeeds is protection against lipid oxidation and the maintenance of the membrane structure since the carotenoids are usually esterified and incorporated into the cell membranes [45]. This positive effect was observed in the present study - fish fed the diets containing the microalgae extracts, especially the diets with carotenoids (D3 and D4), presented a lower MDA $kg^{-1}$ content in the muscle than those fed the control diet. The ingestion of carotenoids via the diet reduced SOD (superoxide dismutase) activity and increased radical scavenging activities in olive flounder [46]. The inclusion of astaxanthin or phycocyanin in diets for *L. calcarifer* larvae raised the resistance against *Vibrio alginolyticus* infection and survival [44]. Thus, it can be said that fish fed the diets with the microalgal extracts may be less susceptible to disease. However, much additional information will be required about those potential effects before a complete understanding.

In conclusion, the present study evidences the suitability to partially substitute fish oil with a lipid saponifiable extract obtained from *Nannochloropsis gaditana* in diets for juvenile *S. aurata* without affecting their growth. This is an improvement in terms of using sustainable ingredients in aquafeeds. The muscle fatty acid profile was altered, specially a reduction of DHA percentage and increment of EPA. Including the non-saponifiable lipid extract did not alter the fish growth parameters nor the muscle fatty acid profile; however, it did increase the concentration of carotenoids in the muscle and reduced the muscle's lipid oxidation. Complementarily, the dietary carotenoids improved skin color, especially yellowness. Therefore, both lipid extracts from the microalga *N. gaditana* could be used, alone or in combination, as dietary ingredients in juvenile gilthead seabream feeding. Further additional studies aimed at assessing the potential long-term effect of these microalgae extracts included in diets for aquacultured fish are required in order to fully ascertain the findings observed in this study.

## Declaration of authors' agreement

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

## CRediT authorship contribution statement

G. Acién-Fernández and R. Sales participated in cultivation of the algae, and purification of microalgal additives. E. Navarro-López and M. C. Cerón-García performed the fatty acid and carotenoid analysis. A. Galafat prepared the aquafeeds. Sáez and T.F. Martínez participated in the feeding trial and performed analysis of lipid oxidation and determination of instrumental color parameters. A. Vizcaíno performed the fish sampling and chemical analysis of fish. R. Sales, M.Y. Tsuzuki and F. J. Alarcón performed the data analysis and drafted the manuscript. M.C. Cerón-García, E. Molina-Grima and F. J. Alarcón designed the work. All authors critically revised and approved the manuscript.

## Declaration of competing interest

The authors declare no conflict of interests.

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*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 Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 06/02/2020/011).

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