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Phaeodactylum tricornutum in finishing diets for gilthead seabream: effects on skin pigmentation, sensory properties and nutritional value

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Abstract Microalgal biomasses are known to play a major role in fish pigmentation, which is particularly important in farmed fish, since colour and external appearance are the first cue for customers when choosing seafood. A study was undertaken to assess the potential of microalgae biomass from the diatom *Phaeodactylum tricornutum* as a functional ingredient for gilthead seabream (*Sparus aurata*) feeds. Three experimental diets were designed: a control diet (CTRL), this same diet supplemented with 2.5% of *P. tricornutum* wild strain (diet MA20); and a third diet with 2.5% of *P. tricornutum* biomass (diet MA37) cultivated under different temperature and light regimes that resulted in higher levels of fucoxanthin. Microalgae diets led to a reduction ($P < 0.05$ in MA37) of whole-body fat and lower lipid retention ($P < 0.05$ in MA20 and MA37). Microalgae did not impact odour, flavour, whiteness, and fatness perception in cooked fillets. Overall, colour analysis showed that *P. tricornutum* biomass led to significant differences compared to control in specific areas: the MA37 diet induced a significantly ($P < 0.05$) lighter and more vivid yellow colouration of seabream operculum ($\Delta E^* \approx 5$) perceptible to the human eye; ventral skin lightness was also affected by the dietary treatments ($P = 0.040$), being higher for microalgae-fed groups, though this difference was not perceptually strong ($\Delta E^* \approx 1.7$). *Phaeodactylum tricornutum* biomass can be used as a functional ingredient, improving external pigmentation and thus contributing to meet consumer expectations in relation to farmed gilthead seabream.

Keywords Gilthead seabream · Microalgae · *Phaeodactylum tricornutum* · Diatom · Skin pigmentation · Quality

Introduction

Being a rich source of important nutrients, including highly digestible proteins, vitamins (A, D, niacin and B_{12}), trace minerals (iodine, selenium) and n-3 polyunsaturated fatty acids (PUFA), fish is considered to be a healthy dietary choice (Kris-Etherton et al. 2002; Larsen et al. 2011; Lund 2013; EFSA 2015). In fish markets where fish is commercialized whole, visual cues such as body shape and skin pigmentation patterns are considered important consumer purchasing criteria (Vasconcellos et al. 2013; Colihueque and Araneda 2014).

Gilthead seabream (*Sparus aurata*) is the major farmed marine fish species in the Mediterranean region (FEAP 2015). Wild specimens show a golden colouration of the forefront, a reddish operculum and a yellow-coloured lateral band, while farmed seabream have thicker skin, which is darker in the dorsal and head areas, and the characteristic iridescent colours are much duller (Grigorakis et al. 2002; Rogdakis

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et al. 2011; Šimat et al. 2012). Like observed for seabream, external appearance can also vary between farmed and wild European seabass (Grigorakis 2007; Arechavala-Lopez et al. 2013). Previous studies have shown that rearing conditions (Flos et al. 2002; Valente et al. 2011) and dietary factors (Wassef et al. 2010) may affect fish pigmentation and external appearance. Fish, like other animals, cannot synthesize carotenoids de novo and therefore depend entirely on dietary sources to achieve their natural pigmentation patterns.

Synthetic carotenoid pigments are commercially available as feed additives, but increasing consumer awareness of synthetic additives has promoted interest in the use of natural carotenoid sources. Microalgal biomass has been successfully tested and may lead to valuable ingredients for the animal feed sector (Shields and Lupatsch 2012), as they are an excellent source of protein, vitamins, trace minerals, long-chain PUFAs (LC-PUFAs) and natural pigments (Leu and Boussiba 2014; Haas et al. 2016). Microalgae carotenoids have been shown to have important biological functions in various fish species (Shahidi and Brown 1998), such as antioxidant properties (Pham et al. 2014; Sahin et al. 2014), acting as immune system modulators (Abdel-Tawwab and Ahmad 2009; Cerezuela et al. 2012a, b; Kim et al. 2013; Reyes-Becerril et al. 2013) and influencing flesh and skin pigmentation (Pham et al. 2014; Sefc et al. 2014). The marine diatom, *Phaeodactylum tricornutum*, is characterized by high levels of n-3 PUFAs, mainly eicosapentaenoic acid (EPA), and high contents of fucoxanthin, an orange-coloured carotenoid (Rebolloso-Fuentes et al. 2001; Peng et al. 2011; Kim et al. 2012). *Phaeodactylum tricornutum* has been successfully used in fish diets with beneficial effects on the seabream immune system (Cerezuela et al. 2012a). However, no literature data exist regarding the effects of *P. tricornutum* on fish quality criteria, such as skin pigmentation and sensory traits.

The objective of this study was to evaluate the potential of two *P. tricornutum* biomasses, differing in their fucoxanthin content, as functional ingredients in finishing diets for gilthead seabream (*Sparus aurata*). Assessment criteria comprised zootechnical growth performance and a detailed characterization of effects on the nutritional value and sensory traits of fillets and skin pigmentation.

Materials and methods

Experimental diets

A control diet (CTRL) was formulated with practical ingredients to contain 45.3% crude protein, 18.6% crude fat and 22.2 MJ kg^{-1} gross energy (dry matter basis). Based on the CTRL formulation, two other diets (MA20 and MA37) were produced. The MA20 diet incorporated 2.5% of *Phaeodactylum tricornutum* wild strain biomass, at the expenses of whole peas. The MA37 diet contained also 2.5% of *P. tricornutum* biomass but with higher levels of fucoxanthin, resulting from different temperature and light regimes used during the cultivation. The strain used was *Phaeodactylum tricornutum* UTEX 640, sourced from the University of Texas (Austin, USA). On a dry basis, the composition of the *P. tricornutum* biomass was crude protein 34%, crude lipid 10%, ash 29% and fucoxanthin 12 mg g^{-1} for MA20 and 16 mg g^{-1} for MA37. The algal biomasses were produced by A4F S.A. (Lisbon, Portugal) in photobioreactors. Diets were isonitrogenous, isolipidic and isoenergetic. Ingredients were ground (below 250 μm) in a micropulverizer hammer mill. Powdered ingredients were then mixed accordingly to the targeted formulation in a double-helix mixer (model 500L, TGC Extrusion, France) to attain a basal mixture. Diets (pellet size 5.0 mm) were manufactured at SPAROS, Lda (Olhão, Portugal) by means of a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 115–120 °C. Upon extrusion, extruded feeds were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling of the pellets, the oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature, but in a cool and aerated location. Samples of each diet were taken for proximate composition analysis (Table 1).

Growth trial

The trial was conducted at the Experimental Research Station of CCMAR (Faro, Portugal). Experiments were directed by trained scientists (following category C FELASA recommendations) and in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei no. 113/2013, de 7 de Agosto) legislation on the protection of animals for scientific purposes. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ("group-1" license by the "Direção Geral de Veterinária", Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Each diet was tested in duplicate groups of 30 seabream with a mean initial body weight of 233 g stocked in 1000 L circular plastic tanks, for 84 days. Fish were fed to apparent satiety, by hand, twice daily during week days, once a day on Saturdays and unfed on Sundays. Excess feeding was minimized and feed intake was quantified throughout the trial. Rearing tanks were supplied with flow-through gravel-filtered, aerated seawater (salinity 34 psu, temperature 19–27 °C, oxygen content of outlet water maintained higher than 5 mg L^{-1}) and subjected to natural photoperiod changes through Summer-Autumn conditions (early August till end-

Table 1 Ingredient and proximate composition of experimental diets

Ingredients (%)	CTRL	MA20W	MA37G
Fishmeal 70 LT[a]	12.0	12.0	12.0
Fishmeal 60[b]	18.0	18.0	18.0
Soy protein concentrate[c]	5.0	5.0	5.0
Wheat gluten[d]	6.0	6.0	6.0
Corn gluten meal[e]	8.0	8.0	8.0
Soybean meal 48[f]	10.0	10.0	10.0
Rapeseed meal[g]	5.0	5.0	5.0
Wheat meal	7.0	5.0	5.0
Wheat: corn DDGS[h]	3.0	5.0	5.0
Whole peas	9.8	7.3	7.3
Fish oil[i]	10.5	10.5	10.5
Palm oil[j]	3.5	3.5	3.5
Vitamin & Mineral Premix[k]	1.0	1.0	1.0
Binder[l]	1.0	1.0	1.0
Antioxidant[m]	0.2	0.2	0.2
Microalgae biomass MA20[n]	0	2.5	0
Microalgae biomass MA37[n]	0	0	2.5
Dry matter (DM), %	96.9 ± 0.0	94.8 ± 0.1	94.6 ± 0.0
Crude protein (% DM)	45.5 ± 0.3	45.4 ± 0.0	45.3 ± 0.2
Crude fat (% DM)	18.6 ± 0.1	18.5 ± 0.2	18.6 ± 0.2
Ash (% DM)	9.9 ± 0.2	10.9 ± 0.1	10.7 ± 0.2
Total phosphorus (% DM)	1.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.1
Gross energy (kJ g^{-1} DM)	22.1 ± 0.0	22.4 ± 0.0	22.4 ± 0.0

[a] Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru

[b] Fair average quality (FAQ) fishmeal: 62% CP, 12% CF, COFACO, Portugal

[c] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands

[d] VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France

[e] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal

[f] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal

[g] Defatted rapeseed meal: 34% CP, 2% CF, SORGAL SA, Portugal

[h] Wheat: corn (80:20) dry distillers' grains with solubles: 33% CP, 2.5% CF, AB Agri, England

[i] COPPENS International, The Netherlands

[j] Crude palm oil: Gustav Heess GmbH, Germany

[k] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings

[l] Kielseguhr (natural zeolite): LIGRANA GmbH, Germany

[m] Paramega PX, Kemin Europe NV, Belgium

[n] Dry biomass of *Phaeodactylum tricornutum*: 34% CP, 10% CF (MA20 with 12 mg g^{-1} fucoxanthin and MA37 with 16 mg g^{-1} fucoxanthin), A4F S.A., Portugal

October). At the end of the trial, fish were slaughtered by immersion in ice-saltwater slurry (4:1) until death. All samplings were done within 24 h following the last meal. At the beginning of the trial, six fish from the initial stock and three fish per tank at the end of the trial were sampled for analysis of whole-body composition. For analysis of quality criteria, after slaughter, fish were packed in insulated polystyrene boxes, with the ventral side upward, covered with plastic and flaked ice and immediately transported to the laboratory. Fifteen fish from each treatment were weighed, scaled and filleted 24 h after death. Left and right fillets (with skin) were separately packed in low-density polypropylene bags (15.2 × 33.0 cm) and kept at 4 °C until sensory assessment by a trained panel.

Biochemical analytical methods

Proximate composition analysis of the diets, whole fish and fillets was made by the following procedures: dry matter by drying at 105 °C for 24 h, ash by incineration of dry sample in a furnace at 550 °C for 12 h, crude protein (N × 6.25) by a combustion technique (at 850 °C) followed by thermal conductivity detection of nitrogen and using LECO FP *528* analyser, crude fat after dichloromethane extraction by the Soxhlet method and gross energy in an adiabatic bomb calorimeter (IKA). Macro minerals (S, Cl, K and Ca), trace minerals (Fe, Cu, Zn, Br and As) and metallic elements (Rb) were measured using spectroscopy X-ray energy dispersive (EDXRF) methodology according to Carvalho et al. (2005). Total lipids in the fillets were extracted according to the method of Folch et al. (1957) and subsequently, the fatty acid composition of fillets was determined by gas-chromatography analysis of methyl esters, according to the procedure of Lepage and Roy (1986), modified by Cohen et al. (1988) and described in detail by Costa et al. (2013). Lipid oxidation in fish fillets was assessed at the time of slaughter (T0) and after 25 weeks of frozen storage at −20 °C, using the polyene index (PI) calculated as the fatty acid ratio: (EPA + DHA)/C16:0 (Šimat et al. 2015). The nutritional contribution (NC) of steam-cooked seabream fillets was calculated as the percentage of the daily adequate intake (DAI) for combined EPA and docosahexaenoic acid (DHA), according to the following formula:

$$\mathrm{NC}\ (\%) = 100 \times \frac{C \times M}{\mathrm{DAI}}$$

where C = EPA+DHA content (mg kg^{-1}), M = typical meal portion consumed (0.160 kg, assuming a 40% fillet yield in a commercial size seabream of 0.400 kg) and *DAI* considered for EPA+DHA was 500 mg day^{-1} for primary prevention of cardiovascular disease in adults (International Society for the Study of Fatty Acids and Lipids [ISSFAL] 2004).

Pigmentation

Skin colour was measured with a tristimulus colorimeter (Macbeth Color-Eye 3000) and the L*, a* and b* coordinates from CIELab system were recorded. Eleven random fish from each treatment were used for this procedure. Colour was determined in triplicate measures in several predefined zones in the fish: the interorbital band (left, medium and right areas), operculum, skin [dorsal, dorsal, intermedium (close to lateral line) and ventral areas] and muscle (dorsal, medium and ventral, corresponding to the skin areas). From a* and b* coordinates, chroma (C*) and hue (H^0) parameters were calculated according to Schubring (2009). To estimate perceptible colour differences (ΔE*) among dietary treatments the CIE76 formula (based on the Euclidian distances between colours in CIELab space) was applied:

$$\Delta E^* = \sqrt{\left(L_2^*-L_1^*\right)^2 + \left(a_2^*-a_1^*\right)^2 + \left(b_2^*-b_1^*\right)^2}$$

For each evaluated zone, average values of colour coordinates were used for ΔE* calculation (CTRL vs MA20, CTRL vs MA37 and MA20 vs MA37). Following the definition of the CIELab colour space and Mahy et al. (1994), distances between colours were considered as being indicative of either an "irrelevant perceptual difference" (ΔE* < 1), a "slight perceptual difference" (1 < ΔE* < 2.3) or a "clear perceptual difference" (ΔE* > 2.3).

Sensory evaluation

A sensory evaluation was carried out in an acclimatized test room equipped with individual booths. The sensory panel was composed of four trained panellists (non-smokers, 50% men, with ages ranging between 40 and 57) from the Portuguese Institute of Sea and Atmosphere, specifically trained in descriptive methods for sensory assessment of wild and farmed fish, according to the guidelines described in Meilgaard et al. (1999) and Martinsdóttir et al. (2009). To reduce the variability within the fillets, the parts close to the head and the tail were rejected. Each fillet was individually wrapped with aluminium foil to avoid odour loss, and then cooked for 10 min at 100 °C in a saturated steam oven (Rational Combi-Master CM6, Cross Kuchentechnik GmbH, Germany). Eight cooked fillets from each treatment were assessed in two independent sensory sessions. In each session four fillets per treatment were presented to the panellists, sequentially, in coded white dishes under normal white lighting (each panellist assessed three fillets, one per treatment). The panellists rated the intensity of sensory attributes on an unstructured line scale (Meilgaard et al. 1999) ranged from 0 cm (absence of attribute) to 12 cm (extremely intense). Results were expressed as the distance (in cm) of each evaluated attribute: odour (typical and atypical), flavour (typical and atypical), whiteness colour and fatness.

Statistical analysis

Except for hue values, data were expressed as means ± standard deviation. All data were subjected to one-way analysis of variance. For ANOVA analysis, parameters expressed as percentages were subjected to arcsine square root transformation. Following ANOVA, means were compared by the Tukey HSD multiple range test. Given that hue is an angular measure, data were treated by a one-way circular ANOVA and group comparison was done by the Watson-Williams test. Statistical significance was tested at 0.05 probability level. In order to assess the possibility of dose-dependent effects, linear models relating "fucoxanthin levels" with the colour variables affected by the experimental factors were fitted by least-squares regression. The hypotheses that fucoxanthin levels have an effect different from zero were assessed via F test ($P < 0.05$). All colour data were also subjected to a correlation analysis (for quality control purposes) and a principal component analysis (with no scaling of variables) prior to the ANOVA. Given that the CIELab colour space is considered approximately perceptually uniform, the lack of variable scaling is required to ensure that the perceptual difference between colours (i.e. the untransformed Euclidian distance in CIELab space) is preserved by the PCA analysis. Statistical analysis was performed using the SPSS (v22, IBM, USA) and R (v3.2.2) statistical software.

Results

At the end of the trial, fish reached a final body weight (FBW) ranging from 413 to 416 g (Table 2). Specific growth rate (SGR) varied between 0.68 and 0.69% day^{-1}, while feed conversion ratio (FCR) ranged from 1.69 to 1.74. Overall growth performance criteria (FBW, SGR, FCR, feed intake and protein efficiency ratio) were not affected ($P > 0.05$) by dietary treatments.

Dietary treatments did not affect ($P > 0.05$) the whole-body protein and phosphorus contents. In contrast, whole-body moisture, fat, ash and energy varied between treatment groups (Table 2). In comparison to the CTRL treatment, seabream fed both microalgae-rich diets showed an increase of whole-body ash content, significant only for MA20 ($P = 0.029$) and a reduction of whole-body fat, yet only significant for MA 37 ($P = 0.020$). Seabream fed diet MA37 presented significantly higher levels of whole-body moisture ($P = 0.038$) and lower energy content ($P = 0.028$) in comparison with CTRL and MA20 groups. Data on weight gain, feed intake and whole-body composition of fish allowed the estimation of nutrient and energy retention. Protein and energy retention were not affected ($P > 0.05$) by dietary treatments. However, the incorporation of

Table 2 Growth performance, whole-body composition and nutrient retention

		CTRL	MA20	MA37	*P* value
IBW[a] (g)		233 ± 2	234 ± 0	233.0 ± 1	
FBW[b] (g)		415 ± 3	413 ± 6	416.0 ± 9	0.907
VFI[c]		1.13 ± 0.02	1.15 ± 0.00	1.13 ± 0.02	0.534
SGR[d]		0.69 ± 0.02	0.68 ± 0.02	0.69 ± 0.02	0.773
FCR[e]		1.69 ± 0.02	1.74 ± 0.05	1.69 ± 0.08	0.552
PER[f]		1.35 ± 0.01	1.33 ± 0.04	1.39 ± 0.07	0.532
Body composition	Initial				
Moisture (%)	63.9	62.8 ± 0.3[a]	62.5 ± 0.1[a]	63.5 ± 0.2[b]	0.038
Protein (%)	18.1	17.8 ± 0.5	18.4 ± 0.0	18.5 ± 0.5	0.278
Fat (%)	13.4	15.4 ± 0.2[b]	13.9 0.3[ab]	13.0 ± 0.6[a]	0.020
Ash (%)	4.5	3.9 ± 0.3[a]	5.0 ± 0.2[b]	4.7 ± 0.1[ab]	0.029
Phosphorus (%)	0.7	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.251
Energy (kJ g^{-1})	9.1	9.5 ± 0.1[b]	9.4 ± 0.0[a]	9.2 ± 0.1[a]	0.028
Retention[g]					
Protein (% intake)		23.4 ± 1.2	25.0 ± 0.7	26.3 ± 2.6	0.365
Fat (% intake)		59.3 ± 2.0[b]	46.6 ± 3.1[a]	42.0 ± 2.5[a]	0.014
Energy (% intake)		27.6 ± 0.1	26.7 ± 0.7	26.1 ± 0.7	0.173

Values are means ± standard deviation ($n = 2$). Different superscripts within a row represent significant differences between treatments ($P < 0.05$)

[a] Initial mean body weight

[b] Final mean body weight

[c] Voluntary feed intake: crude feed intake/(IBW+FBW)/2/84 days

[d] Specific growth rate: (Ln FBW−Ln IBW) × 100/84 days

[e] Feed conversion ratio: dry feed intake/wet weight gain

[f] Protein efficiency ratio: wet weight gain/crude protein intake

[g] Retention: 100 × (FBW × final carcass nutrient − IBW × initial carcass nutrient)/nutrient intake

microalgae (diets MA20 and MA37) led to a significant reduction ($P = 0.014$) of fat retention in comparison with CTRL treatment (Table 2). The biochemical composition of seabream fillets is presented in Table 3. Dietary treatments had no effect ($P > 0.05$) on the protein, lipid and ash content of fish fillets. Similarly, the content of minerals (S, Cl and K), trace elements (S, Cl, K, Fe, Cu, Zn, Br, As) and metal (Rb) were not affected by dietary treatments ($P > 0.05$). In comparison to CTRL fish, those fed the MA37 diet showed a significant reduction ($P < 0.05$) of muscle calcium (Ca) content.

The summarized fatty acid composition of seabream fillets is presented in Table 4. Dietary incorporation of microalgae did not affect the muscle profile in saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Consequently, total levels of n-3 and n-6 fatty acids, its ratio and both thrombogenic (TI) and atherogenic (AI) indexes were also not affected by dietary treatments ($P > 0.05$). Lipid oxidation expressed as the polyene index (PI) showed no differences ($P > 0.05$) between experimental groups, at time of slaughter or after 25 weeks of frozen storage at −20 °C.

Sensory analysis in steam-cooked fillets found no differences ($P < 0.05$) between treatments concerning typical odour, typical flavour, white colour and fatness (Fig. 1). Atypical odours and flavours were not considered relevant by the panel with mean values below 8.8 and 5.6%, respectively (data not showed), of the scale intensity.

The main results of colour measurements are presented in Table 5. Considering colour variables (L*, a*, b*) of the 10 evaluated zones using 11 fish per group, a principal component analysis (PCA) was performed, showing a

Table 3 Proximate composition and mineral contents of seabream fillets

	CTRL	MA20	MA37	*P* value
Protein (%)	20.7 ± 0.6	20.1 ± 1.2	20.4 ± 0.8	0.760
Lipids (%)	6.7 ± 1.2	7.6 ± 1.6	8.3 ± 0.6	0.168
Ash (%)	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.153
S (g kg^{-1})	1.63 ± 0.23	1.56 ± 0.22	1.61 ± 0.25	0.878
Cl (g kg^{-1})	0.42 ± 0.03	0.42 ± 0.08	0.43 ± 0.06	0.961
K (g kg^{-1})	3.64 ± 0.23	3.49 ± 0.27	3.46 ± 0.21	0.452
Ca (g kg^{-1})	0.13 ± 0.03[b]	0.10 ± 0.02[ab]	0.09 ± 0.02[a]	0.039
Fe (mg kg^{-1})	3.44 ± 0.76	3.18 ± 0.55	3.89 ± 0.88	0.346
Cu (mg kg^{-1})	1.10 ± 0.06	1.08 ± 0.06	1.13 ± 0.08	0.476
Zn (mg kg^{-1})	4.02 ± 0.19	3.94 ± 0.36	3.97 ± 0.30	0.908
As (mg kg^{-1})	3.00 ± 0.08	3.06 ± 0.26	3.09 ± 0.26	0.802
Br (mg kg^{-1})	3.10 ± 0.14	3.36 ± 0.36	3.21 ± 0.29	0.382
Rb (mg kg^{-1})	0.60 ± 0.08	0.71 ± 0.06	0.68 ± 0.04	0.059

Values are means ± standard deviation ($n = 5$). Different superscripts within a row represent significant differences between treatments ($P < 0.05$)

Table 4 Summarized fatty acid content of raw fillets and lipid oxidation

Fatty acids[a] (g (100 g)$^{-1}$)	CTRL	MA20	MA37	*P* value
Total SFA[b]	1.76 ± 0.33	1.93 ± 0.48	2.14 ± 0.28	0.299
Total MUFA[c]	1.97 ± 0.41	2.29 ± 0.53	2.46 ± 0.21	0.179
C18:2n-6	0.46 ± 0.10	0.51 ± 0.12	0.57 ± 0.05	0.201
C18:3n-3	0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	0.280
C20:4n-6	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.222
C20:5n-3 (EPA)	0.46 ± 0.09	0.53 ± 0.11	0.55 ± 0.04	0.259
C22:5n-3	0.17 ± 0.03	0.21 ± 0.04	0.22 ± 0.02	0.078
C22:6n-3 (DHA)	0.73 ± 0.13	0.81 ± 0.12	0.85 ± 0.09	0.244
Total PUFA[d]	2.31 ± 0.44	2.65 ± 0.52	2.84 ± 0.18	0.156
Total PUFA n-3	1.63 ± 0.30	1.86 ± 0.34	1.97 ± 0.14	0.180
Total PUFA n-6	0.57 ± 0.12	0.65 ± 0.14	0.72 ± 0.06	0.167
EPA+DHA	1.19 ± 0.22	1.34 ± 0.22	1.41 ± 0.12	0.230
PUFA n-3/n-6	2.88 ± 0.24	2.88 ± 0.17	2.76 ± 0.24	0.604
Atherogenic index (AI)[e]	0.55 ± 0.07	0.52 ± 0.05	0.55 ± 0.07	0.663
Thrombogenic index (TI)[f]	0.27 ± 0.04	0.25 ± 0.02	0.27 ± 0.03	0.701
NC[g] of EPA+DHA (% DAI)	381	429	451	
Polyene index (PI)[h] T0	1.05 ± 0.17	1.08 ± 0.13	1.03 ± 0.17	0.870
Polyene index (PI) T25	0.91 ± 0.08	0.87 ± 0.19	0.82 ± 0.09	0.682

Values are means ± standard deviation (n = 5)

[a] Despite not shown in this summarized format, all identified fatty acids were considered in the composite fractions

[b] SFA: saturated fatty acids

[c] MUFA: monounsaturated fatty acids

[d] PUFA: polyunsaturated fatty acids

[e] AI: (C12:0 + (4 × C14:0) + C16:0)/(total MUFA + total n-3 + total n-6)

[f] TI: (C14:0 + C16:0 + C18:0)/((0.5 × total MUFA) + (0.5 × total n-6) + (3 × total n-3) + ratio n-3/n-6)

[g] NC: nutritional contribution of EPA+DHA, considering a meal portion of 160 g, as % of the daily adequate intake (DAI) of 500 mg day^{-1} for cardiovascular health in adults (ISSFAL 2004)

[h] PI: (EPA+DHA)/C16:0

distinction between control fish and microalgae-fed fish along the first two components (Fig. 2). These two components (which separated control diet fish from microalgae diet fish) accounted for 70% of observed variance, supporting the idea that the dietary inclusion of these microalgae induced a change in fish colouration, compared to the control group. Correlation analysis (Online Resource, fig. S1) confirms the expected consistency between related measurements (e.g. all lightness measurements of the different parts of the muscle are positively correlated), suggesting no technical problems during colour measurements.

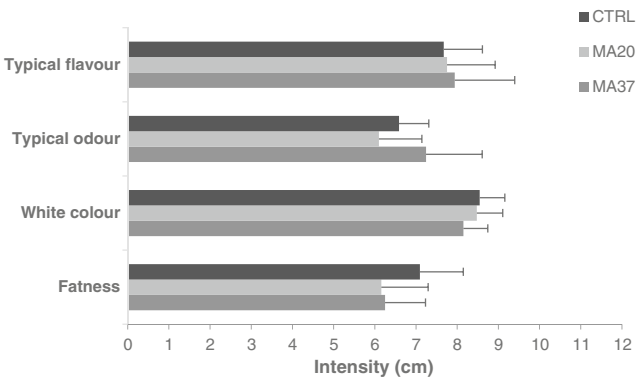

Fig. 1 Sensory scores of seabream steam-cooked fillets by a trained panel. Attribute intensity was rated in an unstructured line scale ranged from 0 (absence) to 12 cm (extremely intense). *Bars* represent mean values ± standard deviation (n = 8)

The interorbital band (left and right values not shown), dorsal intermedium skin (data not shown), dorsal skin and muscle (data not shown) were not affected by the dietary treatments ($P > 0.05$). The skin colour measured in the operculum zone showed that fish fed on the microalgae-rich diets (MA20 and MA37) had significantly higher lightness (L*) values than those fed the control diet (ANOVA, $P = 0.001$). Dietary treatments had no effect on a* values (ANOVA, $P > 0.05$). However, b* and C* values were significantly higher (Tukey HSD, $P = 0.019$ in both cases) in fish fed the MA37 diet than in those fed the control diet. The hue values were close to 90^0, confirming the predominance of a yellow

Table 5 Colour parameters (L*, a*, b*, C*, hue) in the skin of seabream

	CTRL	MA20	MA37	*P* value
Dorsal skin				
L*	64.1 ± 2.0	64.9 ± 2.0	65.4 ± 2.2	0.378
a*	−1.5 ± 0.1	−1.4 ± 0.1	−1.4 ± 0.2	0.590
b*	0.7 ± 0.4	0.8 ± 0.3	1.1 ± 0.6	0.152
C*	1.7 ± 0.2	1.7 ± 0.2	1.9 ± 0.4	0.154
Hue	155.6 (11.4)	151.5 (11.4)	144.9 (16.0)	0.160
Ventral skin				
L*	82.9 ± 2.3	84.4 ± 1.3	84.6 ± 1.3	*0.040*
a*	−1.4 ± 0.1	−1.4 ± 0.1	−1.3 ± 0.2	0.200
b*	1.4 ± 0.3	1.2 ± 0.6	1.7 ± 1.0	0.274
C*	2.0 ± 0.2	1.9 ± 0.5	2.3 ± 0.7	0.253
Hue	135.0 (5.9)	140.5 (11.9)	132.3 (22.4)	0.403
Interorbital band[a]				
L*	60.8 ± 1.9	60.8 ± 2.3	61.5 ± 2.7	0.700
a*	−1.7 ± 0.2	−1.5 ± 0.1	−1.5 ± 0.2	0.071
b*	2.2 ± 0.9	2.1 ± 0.9	2.4 ± 0.7	0.636
C*	2.8 ± 0.7	2.6 ± 0.7	2.9 ± 0.5	0.611
Hue	129.3 (11.7)	128.9 (13.2)	123.6 (8.6)	0.400
Operculum				
L*	74.8 ± 3.7^{a}	79.8 ± 3.2^{b}	79.9 ± 2.7^{b}	*0.001*
a*	0.2 ± 0.3	0.7 ± 0.7	0.8 ± 0.8	0.073
b*	5.3 ± 0.9^{a}	6.5 ± 1.1^{ab}	6.8 ± 1.5^{b}	*0.019*
C*	5.4 ± 1.0^{a}	6.5 ± 1.2^{ab}	6.9 ± 1.6^{b}	*0.019*
Hue	88.0 (3.0)	84.5 (5.2)	83.8 (5.21)	0.083

Values are means ± standard deviation (n = 11). For hue values, the mean angle and the circular standard deviation are provided. Different superscripts within a row represent significant differences between treatments ($P < 0.05$)

[a] Interorbital band values refer to the medium zone, left and right areas data are not shown

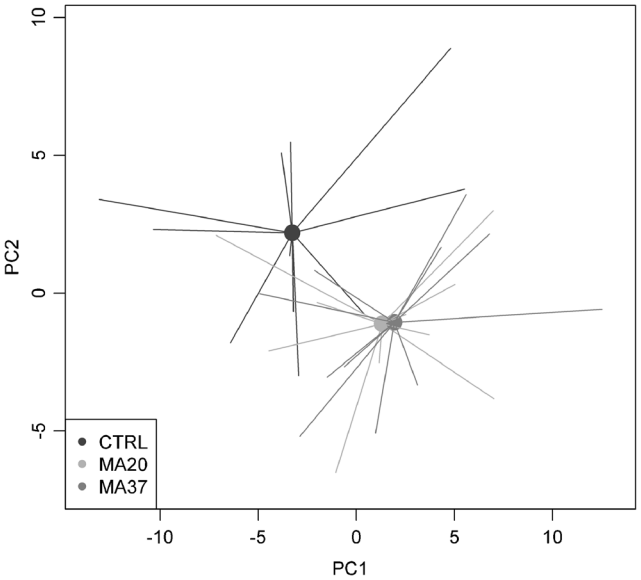

Fig. 2 Principal component analysis score plot, showing the similarity between fish pigmentation when taking into account all colour measurements performed (operculum, interorbital band, dorsal, intermedium and ventral skin and muscle). Values represent projections of the samples (i.e. fish) onto the first two principal components, connected by a line to the corresponding centroid, obtained from a PCA analysis (no scaling) of all the colour measurements (L*, a* and b*) for all measured zones in each fish (n = 11 per dietary treatment). Zones considered were as follows: operculum, interorbital band (left, medium and right), skin (dorsal, intermedium and ventral) and muscle. These two components accounted for 70% of the variance observed across the 30 variables. Samples are coloured according to the corresponding dietary treatment

colouration in the operculum. Although hue values in operculum were not significantly different between the three treatments following circular ANOVA ($P > 0.05$), a trend can be perceived, since the mean hue of fish fed the MA37 diet was significantly different from that of control fish (Watson-Williams test, $P = 0.032$). Fitting a linear model relating "opercular hue" and "fucoxanthin level" also supports the notion that there might be a real (F test, $P = 0.026$), though small, dose-dependent effect of microalgae on opercular hue. Overall, this study shows a clearly perceptible difference between control and microalgae groups at the operculum ($\Delta E^* \approx 5$) level. A substantial effect of the dietary treatments on ventral skin lightness was also noted ($P = 0.040$), showing increased values for the microalgae groups, though pairwise differences between groups (Tukey HSD) were never significant due to high variability in the control group. Nevertheless, fitting a linear model relating "ventral skin lightness" and "fucoxanthin level" supports the existence of a dose-dependent effect (F test, $P = 0.019$) that would explain the slight perceptual differences observed between the ventral skin colour of control and microalgae-fed groups ($\Delta E^* \approx 1.7$).

Discussion

Alongside the well-established applications of microalgae in aquaculture hatcheries, there is currently a drive to exploit the use of algal biomass in formulated animal feeds, both for aquaculture species and terrestrial livestock, as this may provide specific nutritional and physiological benefits during different periods of the life cycle (Shields and Lupatsch 2012; Chauton et al. 2015). However, the high costs of algal biomass compared to commodity feedstuffs currently confine their commercial use to niche applications. A beneficial effect on fish quality criteria is a potential application of microalgae in aquaculture feeds. Quality is a broad and dynamic concept that is dependent on consumer perception of a food product (Grunert 1995). Several definitions may be drawn from published data, but often quality is associated with intrinsic and extrinsic cues that are used by consumers to form their perception of food

quality (Oude Ophuis and Van Trijp 1995). Intrinsic traits are linked with the physicochemical characteristics of food such as nutrient content, flavour, sensory properties, and food safety, among others, while extrinsic traits concern the imaging, branding, packaging and cost. In the present work, several intrinsic traits, like omega-3 profile, lipid oxidation, sensory evaluation and colour, were measured to infer if a dietary supplement of *P. tricornutum* could improve the quality of farmed seabream.

Our data show that 2.5% dietary supplementation of *P. tricornutum*, with variable levels of fucoxanthin, did not impair growth and feed efficiency of seabream. In general, the inclusion of microalgae biomass at low levels leads to no differences, or even improved results when compared with commercial or control feeds. In gilthead seabream juveniles, the supplementation of 5 and 10% *P. tricornutum* (Cerezuela et al. 2012a) and up to 20% supplementation of *Scenedesmus almeriensis* (Vizcaíno et al. 2014) showed no differences on specific growth rate, with beneficial effects observed on the immune system response and intestinal function, respectively. Other studies performed in seabream showed that neither growth nor feed efficiency were affected by the dietary inclusions of 4% *Haematococcus pluvialis* (Gomes et al. 2002) and 6% of *Chlorella vulgaris* (Gouveia et al. 2002). In our study, both microalgae-fed groups presented a reduction of whole-body fat content and consequently lower dietary fat retention. This reduction of body fat content probably occurred at the level of perivisceral fat (a preferential fat deposition site in seabream), since no differences were found on the fillet fat content. A lipid lowering effect of microalgae-rich diets was previously observed. Reduction of whole-body fat associated with dietary inclusion of microalgae was described in Japanese flounder (Kim et al. 2002), in common carp (Nandeesha et al. 1998; Kiron et al. 2012) and Atlantic salmon (Kiron et al. 2012). *Spirulina* supplementation was shown to increase hepatic carnitine palmitoyltransferase activity and hepatic carnitine level, inducing lipid mobilization and reduction of lipid accumulation in red seabream (Nakagawa et al. 2000). Similarly, an activation of the lipid metabolism by hormonal regulation induced by dietary algae was also previously mentioned (Nematipour et al. 1987, 1990). Though several explanations have been put forth, the mechanisms underlying this effect are not completely understood. Fucoxanthin, the major carotenoid present in *P. tricornutum* biomass has been associated with lower accumulation of abdominal white adipose tissue in rodents. In these studies, fucoxanthin was linked to a depression of lipogenic enzyme activity and an increase in fatty acid oxidation (Peng et al. 2011; Ha and Kim 2013; Maeda 2015). Our data shows a (non-significant) trend towards a higher magnitude on the whole-body lipid lowering effect with the MA37 biomass, which contained higher fucoxanthin levels than the MA20 biomass, suggesting that the lipid lowering effect of fucoxanthin might be dose-dependent.

The nutritional value of farmed fish is largely associated with its fatty acid profile, more specifically with its content in omega-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFAs), because of its physiological importance in human health (Kris-Etherton et al. 2009). In general, the fatty acid composition of fish fillets tends to mimic dietary composition. *Phaeodactylum tricornutum* is known to have relatively high levels of EPA (Fajardo et al. 2007). However, given the low dietary microalgae incorporation levels tested (2.5%), dietary EPA levels were similar among the various experimental diets. Consequently, microalgae-rich diets did not affect the seabream fillet profile in SFA, MUFA and PUFA. Total levels of omega-3 (n-3) and omega-6 (n-6) fatty acids, their ratio and both TI and AI were also not affected by the inclusion of microalgae. In our study, raw gilthead seabream fillets showed total EPA and DHA levels ranging from 1.19 g·(100 g)$^{-1}$ in fish fed the CTRL diet, 1.34 and 1.41 g·(100 g)$^{-1}$ in those fed MA20 and MA37 diets, respectively. The consumption of a 160 g portion of seabream fillet would represent 381, 429 and 451% (for CTRL, MA20 and MA37 treatments, respectively) of combined EPA and DHA daily adequate intake primary prevention of cardiovascular disease in adults (ISSFAL 2004). Since fish is not generally consumed on a daily basis, calculations for a weekly intake seem more appropriate. Consumption of seabream fillets twice a week would cover 109–129% of the adequate EPA + DHA intake for enhanced cardiovascular health. The presence of highly unsaturated fatty acids increases the fillets' susceptibility to lipid oxidation over time. Lipid oxidation originates undesirable off-flavours and unhealthy compounds such as free radicals and reactive aldehydes, which are considered particularly unpleasant by consumers (Frankel 2005). The polyene index used to measure lipid oxidation in seabream fillets revealed no differences between treatments at time of slaughter or after 25 weeks of frozen storage at −20 °C. Microalgae antioxidant potential and its application in food preservation have been described (Rodriguez-Garcia and Guil-Guerrero 2008). Enhanced resistance to lipid oxidation was reported in oil: water emulsions containing microalgae by Gouveia et al. (2006). The previously described antioxidant effect associated with dietary algae biomasses (Goiris et al. 2012), and in particular due to the presence of high fucoxanthin levels (Rodriguez-Garcia and Guil-Guerrero 2008; Peng et al. 2011), was not shown to have any preventive effect on lipid oxidation during storage, in our study.

Results from sensory evaluation by a trained panel showed that *P. tricornutum* supplemented diets did not affect the organoleptic properties of steam-cooked seabream fillets. Literature refers similar results for channel catfish fed *Schizochytrium* sp. diets (Li et al. 2009), common carp fed *Spirulina* diets (Nandeesha et al. 1998) and European seabass fed *Isochrysis* sp. diets (Tibaldi et al. 2015). A recent study with a DHA-rich *Schizochytrium* sp. reported no effect of microalgae incorporation levels on instrumentally measured

texture criteria and water holding capacity of Atlantic salmon fillets (Kousoulaki et al. 2015).

Consumers' acceptance of a food product is highly conditioned by its appearance. The influence that colour and visual image may exert on flavour perception and food acceptability for different food products has been well described (Hutchings 1999; Spence et al. 2010). Dietary carotenoids exert a primary role on fish skin and muscle pigmentation, and are responsible for the typical colour of many important seafood products. Fish are unable to synthesize carotenoids de novo, but they are capable of modifying and metabolizing dietary carotenoids (Goodwin 1984; Shahidi and Brown 1998; Sefc et al. 2014). The colour of fish and shellfish products affect consumer acceptance and market value (Sacton 1986; Vasconcellos et al. 2013). Carotenoid deposition is not only influenced by the fish species and carotenoid source and chemical composition, but is also largely dependent on the organs and tissues considered (reviewed by Shahidi and Brown 1998). There is a large body of literature describing carotenoids' metabolism, deposition and their role on skin and flesh pigmentation in salmonids (Torrissen 1985; Storebakken et al. 1987; Bjerkeng 2000). Experimental studies targeting skin pigmentation of the *Sparidae* species, like red porgy or gilthead seabream, are much scarcer. In red porgy, skin pigmentation was successfully improved with the use of microalgae at a dietary inclusion of 5% *Spirulina* and 3.3% *Haematococcus* (Chatzifotis et al. 2011) and with shrimp shell meal (Kalinowski et al. 2005). Conversely, in studies performed with gilthead seabream using dietary microalgae *Chlorella* and *H. pluvialis*, an increase in skin carotenoid deposition level was observed, although this was not reflected as an improvement in skin pigmentation (Gomes et al. 2002; Gouveia et al. 2002). Colouration is determined by the specific carotenoids used and the carotenoid composition (Bjerkeng 2000), and its concentration alone cannot be used as a criterion of perceived colour (Little et al. 1979). The usual dimensions of perceived colour are hue, chroma and lightness, usually instrumentally measured as L* lightness, a* redness and b* yellowness (CIE 1976). Considering colour data from all measured zones (using PCA analysis), a clear difference was found between control and microalgae groups. However, differences found with PCA were attributed to dissimilar skin areas between groups, particularly at operculum level, where higher lightness values were found in both microalgae-fed groups, compared to the control treatment. The operculum hue values were close to 90^0, confirming the predominance of a yellow colouration in all groups. Hue values were significantly different in fish fed the MA37 diet compared to those fed the control diet. Moreover, using diet MA37 induced a lighter, more vivid yellow colouration of seabream operculum (higher b* values) and higher chroma (C*) compared to control fish. This is probably associated with the high levels of fucoxanthin, an orange-coloured pigment in MA37, since these differences were not found between CTRL and MA20 groups. On the other hand, the use of *P. tricornutum* biomass did not affect the skin pigmentation pattern in the interorbital band and dorsal zones of seabream. Using a consumer-type approach to a hedonics appearance assessment, 15 out of 16 untrained volunteers preferred the microalgae supplemented groups, compared to the control group. This preliminary information may be used as an indication for future consumer studies. In a recent study by Tibaldi et al. (2015) in which European seabass was fed *Isochrysis*-rich diets, it was found that skin lightness (L*) was not affected by dietary treatments. However, there was a significant increase of greenness (a*) in the dorsal skin of fish fed the diet with the highest level of microalgae. This was coupled with increased hue values and slightly different colour saturation (chroma). An enhanced greenish skin pigmentation had already been described in European seabass juveniles fed *Tetraselmis suecica* (Tulli et al. 2012) and in Atlantic cod fed a mixture of *Nannochloropsis* sp. and *Isochrysis* sp. (Walker and Berlinsky 2011). In red porgy, a diet containing *H. pluvialis* resulted in reddish skin colouration, while diets with *Spirulina* and alfalfa promoted a yellowish colouration (Chatzifotis et al. 2011). Altogether, these studies demonstrate that natural carotenoids from microalgae can be used as tools to tailor the skin pigmentation in fish. However, further studies are needed to establish and understand the efficiency of the various carotenoid types, since the pathways regulating skin pigmentation are species-specific.

Overall data from our study show that *P. tricornutum*, a microalga rich in fucoxanthin, when incorporated at 2.5% in finishing diets for gilthead seabream resulted in a reduction of whole-body fat and originated a lighter and more vivid yellow colouration of seabream operculum and a higher lightness of ventral skin. Farmed fish require adequate pigmentation patterns to respond to consumer demands. Colour is the first quality attribute used by consumers, impacting the visual assessment and freshness perception of fish, which are key purchase determinants. Incorporation of microalgae pigments in aquafeeds also gives aquaculture products a more natural-like image, reducing any impression of manipulation that supplementation may suggest. Consumers often associate synthetic additives in foods with higher health risks, while natural additives are generally perceived as better and more wholesome (Devcich et al. 2007; Dickson-Spillmann et al. 2011). The dietary use of microalgal biomasses may have benefits that go beyond fulfilling the basic nutritional needs of the animal, and aspects such as improvement of the external pigmentation can contribute to consumers' expectations towards farmed fish being met.

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