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Effects of Fish Oil and Microalgae Finishing Diets on Fillet Fatty Acid Recovery, Liver and Intestine Histology, and Innate Immune Parameters of European Sea Bass (*Dicentrarchus labrax*) Fed with Rapeseed Oil

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Abstract

This study evaluated the use of finishing diets to reduce fish oil (FO) inclusion in European sea bass feeds while preserving fillet quality and n-3 fatty acid content. Fish (197 g) were fed for 25 weeks with a control FO diet, based on 9% FO and 6% plant oil, or diets where 6% FO was replaced by rapeseed oil (RO) or microalgae (AO; *Nannochloropsis* sp. and *Schizochytrium* sp.). After 13 weeks, 2 groups of fish fed the RO diet were switched to the FO or AO finishing diets for the final 12 weeks. Growth, feed efficiency, body composition, and health were unaffected by dietary treatments. However, long-term RO feeding reduced eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) and increased monounsaturated and n-6 fatty acids compared to fish fed AO or FO. A 12-wk FO finishing diets shifted morphometric traits, fatty acid profiles, liver histology, and overall fish quality of fish previously fed with RO toward those of fish continuously fed FO, whereas the AO finishing diet produced a smaller shift. Longer finishing periods may further enhance fillet quality when using microalgae-based oils.

Keywords: wash-out; polyunsaturated fatty acids; fish; growth performance indexes; lipid quality indexes; liver; intestine; histology; immunology

Key Contribution: Long-term RO feeding strongly reduced n-3 PUFA (EPA, DHA) and lipid quality indexes (Atherogenic Index, AI; Unsaturation Index, UI; and fish lipid quality, FLQ) compared to FO and AO. The FO finishing diet almost fully restored the fatty acid profile and fillet quality, while AO only partially improved them.

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1. Introduction

Aquaculture has expanded rapidly over the last decades and now supplies more than half of the fish consumed globally, increasing the demand for nutritionally balanced and sustainable aquafeeds [1]. Traditionally, fishmeal (FM) and fish oil (FO) have been the

primary protein and lipid sources in aquafeeds because of their high digestibility, balanced amino acid profile, and richness in essential long-chain n-3 polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [2,3]. However, the continued dependence of aquaculture on marine-derived feed ingredients places significant pressure on wild fisheries and raises concerns regarding the long-term sustainability, availability, and cost of FM and FO [4,5]. Consequently, the aquafeed industry has increasingly explored alternative and more sustainable ingredients, including plant-derived oils and meals, terrestrial animal by-products, insects, and microalgae [3,6,7].

Among these alternatives, vegetable oils have been widely adopted as substitutes for FO due to their availability, lower cost, and favourable environmental profile. Several studies and recent meta-analyses have demonstrated that partial or even complete replacement of FO with plant oils can maintain acceptable growth performance in many fish species, although responses are species-dependent [3]. In Mediterranean marine fish species such as European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), high levels of FO replacement by plant oils have often resulted in reduced growth performance and feed efficiency, or alterations in tissue lipid composition although some gradual adaptation to plant oil occured after long-term feeding [8–16]. These limitations are mainly associated with the low levels of EPA and DHA in vegetable oils and the limited capacity/inability of marine fish, particularly European sea bass, to biosynthesize LC-PUFA from C18 fatty acid precursors through desaturation and elongation activity [8,14,17,18]. As a result, prolonged feeding with plant oil-based diets generally decreases the EPA and DHA content of fish tissues and lowers the nutritional value of fillets for human consumers [19].

To address this challenge, two main nutritional strategies have emerged. The first involves the use of alternative lipid sources naturally rich in long-chain polyunsaturated fatty acids (LC-PUFA), particularly microalgae-derived oils. The second consists of "finishing" or "wash-out" feeding strategies, in which fish are initially reared on diets low in marine ingredients and subsequently fed diets rich in LC-PUFA before harvest to restore the fatty acid profile of the fillet. Finishing strategies using FO have been successful in several aquaculture species, including salmonids, seabream, and sea bass, demonstrating partial or complete recovery of tissue EPA and DHA levels after periods of vegetable oil feeding [12,19–40]. Nevertheless, the reliance of these approaches on FO during the finishing phase does not fully resolve sustainability concerns.

Microalgae have recently attracted considerable attention as promising sustainable alternatives to FO because they constitute the primary natural producers of marine LC-PUFA. Several species, including *Nannochloropsis* sp., *Phaeodactylum tricornutum*, and *Schizochytrium* sp., are particularly rich in EPA and/or DHA and additionally provide valuable bioactive compounds such as pigments, antioxidants, vitamins, and polysaccharides [41,42]. Beyond their nutritional value, microalgae cultivation may contribute to reducing the environmental impact of aquafeeds through lower dependence on wild fish stocks, lower contaminant accumulation, and potential carbon dioxide mitigation during production [19,43,44]. Consequently, microalgae-derived ingredients have been increasingly investigated as partial replacements for FM and FO in aquafeeds [41,45,46]. In European sea bass and gilthead seabream, previous studies have demonstrated that partial replacement of dietary FO with microalgal biomass or oils can maintain growth performance and improve tissue fatty acid quality depending on the inclusion level and algal species used [45,47–49].

Despite these promising findings, important knowledge gaps still exist regarding the application of microalgae-based finishing diet strategies in marine fish nutrition. Most previous studies have evaluated microalgae as a direct FO replacement throughout the production cycle or as finishing diets in large adults. Compared to the numerous finishing diet studies based on FO diets, to our knowledge, only three such studies used finishing

diets based on microalgae and these were all short-term studies (42–90 days) [50–52]. They either applied finishing diets in formulations still containing relatively high levels of FO and microalgae were used as additives in FO-based diets [51,52] or they focused on the replacement of plant oil rather than FO [50]. They all focused on the finishing phase in large adult fish but lacked a first growing phase based on plant oil based diets. Moreover, limited information is available on the combined use of EPA-rich and DHA-rich microalgae in finishing diets specifically formulated to restore fillet LC-PUFA levels following prolonged feeding with plant oil-based diets. Furthermore, the effects of such strategies on growth performance, nutrient utilization, tissue fatty acid recovery, and overall health in European sea bass remain largely unexplored.

Therefore, the present study aimed to compare the long-term dietary effects of two microalgae-based ingredients, the EPA-rich *Nannochloropsis* sp. and the DHA-rich *Schizochytrium* sp., with those of FO or rapeseed oil (RO) in European sea bass diets. Given the limited availability of FO and the relatively high cost of microalgae, the study also investigated the feasibility of further reducing dietary inclusion of FO and AO through a finishing ("wash-out") feeding strategy. In this approach, fish initially reared on a RO-based diet low in marine ingredients were subsequently fed diets containing FO or AO in order to restore fillet nutritional quality, while simultaneously reducing reliance on finite marine resources and improving the sustainability of aquafeed formulations. The effects of replacing FO with RO or AO, either throughout the entire feeding period or exclusively during the finishing phase, were evaluated in terms of growth performance, feed utilization, fillet proximate composition and fatty acid profile, liver and posterior intestine histology, and innate immune responses.

2. Materials and Methods

2.1. Animal Ethics Statement

All experiments involving fish were conducted in accordance with EU guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU) approved by the Greek presidential decree (ΠΔ56/2013). The research followed the generally accepted "3Rs" (replacement, reduction, refinement of animal used) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (https://arriveguidelines.org). The Hellenic Centre for Marine Research (HCMR) rearing facilities are licensed by the Greek authorities (EL25-BIO-37 and EL91-BIOexp-04). The experiment was designed in accordance with the Ethics Committee of the Attica region council, Greece (protocol 2919-13/06/2018) and the ARRIVE guidelines. It was supervised by a scientist (MM) accredited by the Federation for Laboratory Animal Science Associations (FELASA, Functions A–D) who was responsible for the care, anesthesia, sampling and euthanasia of experimental fish.

2.2. Microalgae

Two microalgae meals were chosen for their richness in n-3 PUFA. *Nannocholoropsis* sp. (21.00% lipids), rich in EPA (21.33% of total FA) and also containing arachidonic acid (ARA, 20:4 n-6, 5.60% of total FA), was obtained from Algatechnologies Ltd. (Hevel Eilot, Israel) under the name AlgaNea. *Schizochytrium* sp. (51.55% lipids), chosen for its richness in DHA (52.76% of total FA), was obtained under the name Algaprime (Corbion, Amsterdam, The Netherlands). Their proximate composition is given in Table 1.

Table 1. Proximate composition (% as-is) and EPA-DHA contents (% of total fatty acids) of the microalgae used in the experimental diet AO.

	***Nannochloropsis* sp.**	***Schizochytrium* sp.**
Moisture (%)	4.24	3.23
Ash (%)	8.80	5.66

Table 1. *Cont.*

	***Nannochloropsis* sp.**	***Schizochytrium* sp.**
Proteins (%)	35.36	12.79
Lipids (%)	21.00	51.55
20:5 n-3 (EPA)	4.48	0.22
22:6 n-3 (DHA)	0.00	27.20

AO: microalgae-based diet; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.

2.3. Experimental Diets

Three isoenergetic (21.5 MJ/Kg), isolipidic (17.4%), and isonitrogenous (44.1% CP) diets were formulated based on FM and a mixture of plant ingredients commonly used for European sea bass aquafeeds (Table 2): an FO-based control diet similar to commercial diets (9% FO, 6% RO, diet FO), a plant oil-based diet containing only 3% of FO to cover the basic DHA/EPA requirement and 12% of RO (diet RO), and a diet based on a mixture of the 2 microalgae meals each providing 1.5% of the dietary lipids content (diet AO, 7.2% *Nannochloropsis* sp., 3% *Schizochytrium* sp., 3% FO and 9% RO). They were produced at the facilities of the Applied Nutrition Laboratory of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of HCMR in Anavyssos, Greece. All ingredients, except for 80% of the dietary oils (FO and RO), were mixed together and 4.5 mm pellets were obtained using a floor-standing food mixer (Hobart, Troy, OH, USA). The pellets were dried at 40 °C for 6 h and the remaining dietary oil was added through vacuum coating (Dinnissen, Sevenum, The Netherlands). The three diets were analyzed for proximate and fatty acid composition (Tables 2 and 3) as described in Section 2.6.

Table 2. Formulation and proximate composition of the 3 experimental diets for European sea bass (%, as fed basis).

	FO	**AO**	**RO**
Ingredients			
Fish meal 68 [1]	17.00	17.00	17.00
Wheat meal	16.74	13.69	16.69
Wheat gluten	9.00	9.00	9.00
Corn gluten	15.00	15.00	15.00
Soybean meal 47 [1]	9.00	9.00	9.00
Soybean concentrate 60 [1]	14.50	10.30	14.50
Fish oil	9.00	3.00	3.00
Rapeseed oil	6.00	9.00	12.00
Nannochloropsis sp.	0.00	7.20	0.00
Schizochytrium sp.	0.00	3.00	0.00
Mono-calcium phosphate [2]	2.50	2.50	2.50
Mineral and vitamin premix [2,3]	0.25	0.25	0.25
Vitamin C [2]	0.06	0.06	0.06
Lysine [2]	0.50	0.50	0.50
Methionine [2]	0.30	0.30	0.30
Taurine [2]	0.15	0.20	0.20
Proximate composition			
Moisture [4]	7.10	7.33	7.00
Ash [4]	6.78	7.13	6.93
CP [4]	43.95	44.04	44.18
EE [4]	17.22	17.50	17.47
NFE [5]	24.96	24.12	24.41
OM [5]	86.12	85.67	86.07
CF [6]	1.58	1.77	1.57
ADF [6]	4.15	4.80	4.12

Table 2. *Cont.*

	FO	AO	RO
NDF [6]	2.15	2.56	2.14
Energy (MJ/kg) [7]	21.49	21.48	21.54

FO = fish oil; AO = algal oil; RO = rapeseed oil. [1] The numbers next to the ingredients (fishmeal, soybean meal, and soybean concentrate) represent their protein content. [2] Mono-calcium phosphate, lysine, methionine, vitamin C (ascorbic acid, Stay-C) (Irida, Parga, Greece), taurine (MilliporeSigma, Burlington, Massachusetts, USA), and the premix were diluted with corn gluten to 100 g. [3] Per kilogram of mineral and vitamin premix (Irida, Greece): vitamin A (retinyl acetate, 4,000,000 IU), vitamin D3 (cholecalciferol, 800,000 IU), vitamin E (DL-α-tocopherol, 100 g), vitamin C (Stay-C, 80 g), vitamin B1 (thiamin, 8 g), vitamin B2 (riboflavin, 8 g), vitamin B3 (niacin, 60 g), vitamin B5 (D-pantothenic acid, 24 g), vitamin B6 (pyridoxine-HCl, 8 g), vitamin B7 (biotin, 400 mg), vitamin B8 (inositol, 60 g), vitamin B12 (cyanocobalamin, 80 mg), vitamin K3 (menadione, 4 g), folic acid (1.6 g), iron ($FeSO_4$, H_2O, 28 g), manganese (MnO, 14 g), iodine (KI, 2.4 g), copper ($CuSO_4$, 5 H_2O, 2.8 mg), zinc ($ZnSO_4$, H_2O, 24 g), and 2 antioxidants (16 mg of butylated hydroxyanisole, BHA, E320 and 160 mg of butylated hydroxytoluene, BHT, E321 in carrier $CaCO_3$). [4] CP = crude protein, EE = ether extract, moisture, and ash are all analyzed concentrations. [5] Nitrogen-free extract and organic matter were calculated using the formulae NFE = 100−(crude protein + crude lipid + moisture + ash); OM = 100−moisture−ash. [6] Crude fiber, acid detergent fiber, and neutral detergent fiber were calculated based on the ingredients' CF, ADF, and NDF content. [7] Energy was calculated using the formula (CP × 23.6 + EE × 39.5 + NFE × 17.3)/100.

Table 3. Fatty acid profile (% total fatty acid) of the 3 experimental diets for European sea bass.

Fatty Acids	FO	AO	RO
14:0	2.79	1.64	1.22
15:0	0.43	0.21	0.18
16:0, PA	14.31	14.17	9.93
17:0	0.50	0.27	0.24
18:0, SA	3.61	2.51	2.60
20:0	0.64	0.53	0.59
22:0	0.44	0.42	0.46
24:0	0.27	0.23	0.26
Σ SFA	23.00	20.02	15.49
16:1 n-7, POA	3.47	3.74	1.61
16:1 n-9, HDA	0.35	0.18	0.14
17:1n-7	0.21	0.16	0.13
18:1 n-7	2.70	2.53	3.03
18:1 n-9, OA	31.60	35.40	43.34
20:1n-7	0.13	0.08	0.09
20:1 n-9	1.10	0.98	1.20
20:1n-11	0.14	0.10	0.10
22:1n-9	0.29	0.25	0.30
22:1 n-11, EA	0.60	0.38	0.44
24:1 n-9	0.71	0.43	0.50
Σ MUFA	41.32	44.25	50.90
Σ n-9 MUFA	34.05	37.25	45.48
18:2 n-6, LA	14.00	16.04	19.83
18:3 n-6	0.12	0.11	0.07
20:2 n-6, EDA	0.22	0.13	0.17
20:3 n-6, ERA	0.20	0.19	0.13
20:4 n-6, ARA	0.66	0.90	0.37
Σ n-6 PUFA	15.20	17.37	20.57
18:3 n-3, ALA	3.46	4.10	5.26
18:4 n-3, SDA	0.77	0.41	0.40
20:3 n-3	0.17	0.11	0.16
20:4 n-3	0.32	0.25	0.20
20:5 n-3, EPA	4.76	3.87	2.31
22:5 n-3, DPA	0.59	0.34	0.32
22:6 n-3, DHA	10.40	9.28	4.40
Σ n-3 PUFA	20.48	18.35	13.05

Diets FO = fish oil; AO = algal oil; RO = rapeseed oil; ALA = α-linolenic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; DPA = Docosapentaenoic acid; EA = erucic acid; EDA = eicosadienoic acid; EPA = eicosapentaenoic acid; ERA = eicosatrienoic acid; HDA = hypogenoic acid; LA = linoleic acid; MUFA = monounsaturated fatty acids; n-3/n-6 PUFA = ratio of omega 3/omega 6 FA; OA = oleic acid; PA = palmitic acid; POA = palmitoleic acid; PUFA = polyunsaturated fatty acids; SA = stearic acid; SDA = Stearidonic acid; SFA = saturated fatty acids; Σ = sum.

2.4. Fish Rearing

Adult fish fed a commercial diet (Gorgo 4.5 mm, 45% crude protein and 17% lipids, containing FM, FO, wheat, sunflower meal, soya protein concentrate, soybean meal and corn gluten, Zoonomi, Vrahati Korinthias, Greece) were transferred from the HCMR sea cage facilities in Souda (Crete, Greece) to the IMBBC Aqualabs rearing facilities in Gournes (Crete, Greece). Fish were acclimated for 10 d in a large 5 m^3 tank (2.4 × 1.7 × 1.2 m, L × l × H) in a sheltered outdoor facility. They were fed ad libitum twice a day with the same commercial diet mentioned above. After the acclimation period, 240 European sea bass with an initial body weight of 197.5 ± 35.4 g were randomly distributed in 12 nets of 840 L (1.4 × 0.5 × 1.2 m, L × l × H) in 4 cement tanks of 5 m^3 (3 nets/tank). The fish were kept in an open flow system supplied at a high renewal rate of 40%/h with borehole seawater (salinity: 34‰) enabling a stable temperature of 20.0 ± 0.6 °C and natural photoperiod. The physico-chemical properties of the water remained stable throughout the experiment (pH: 7.80 ± 0.22; O_2: 8.3 ± 0.5 ppm; oxygen saturation: 91.0 ± 5.6%). Fish were fed ad libitum until the fish reached apparent satiation (as indicated by the cessation of surface feeding and their return to the bottom of the nets), twice daily (at 9:00 and 15:00). In the rare case of uneaten pellets falling below the nets, they were counted and their weight (evaluated from the average weight of 50 pellets determined for each diet) was subtracted to accurately determine feed intake.

A block randomized design was used to obtain 3 large tanks each including all 3 basal diets assigned randomly to the 3 nets and 1 technical non-factorial validation tank with 3 nets of fish fed a single diet (RO) for the 13 wk-growth period (see graphical abstract and Section 2.9). Therefore, fish fed the RO-based diets were kept in 6 nets (1 net in each of the 3-block design tanks and 3 nets in the validation tank), while fish fed the FO- and AO-based diets were kept in triplicate nets. At the end of the 13 wk-growth period, fish were sampled and assigned to new diets in duplicate nets for the finishing period of the study, which lasted for an additional 12 wk (25 wk in total). Fish fed the FO or AO diets continued to be fed the same diets. Fish fed the RO diet were either switched to FO or AO based diets or continued being fed on the RO diet (see graphical abstract). Fish were fed twice daily to satiation and the distributed feed was weighed daily for each net.

2.5. Samplings, Growth Performance, Morphometric Indices

Sampling was performed after 13 wk (growth period) and 25 wk (finishing period) of the feeding trial on 24 h-fasted fish. Fish were randomly sampled, anesthetized with 0.05% clove oil (diluted 1:10 in ethanol), and individually weighed to determine growth performance, feed efficiency, and survival. Six fish were sampled per dietary treatment to determine the proximate composition and fatty acid profile of the fish fillets (2 fish per triplicate net at the end of the growth period and 3 fish per duplicate net at the end of the finishing period). Livers and intestines were dissected from 3 fish/net and stored in 20 volumes of 4% formalin for histological analysis. At the end of the finishing period, liver, viscera, and visceral fat were collected from 12 fish per dietary treatment (6 fish per replicate net) and individually weighed to determine hepatosomatic (HSI), viscerosomatic (VSI), and mesenteric fat index (MFI). Blood samples were also collected at 25 wk, from 8 fish per net (16 fish per dietary treatment), through caudal puncture using 2.5 mL syringes without heparin. A 20 µL blood sample was heparinized for hemoglobin analysis. Non-heparinized blood was allowed to clot for 3 h at 4 °C and centrifuged at 16,000× *g* for 10 min at 4 °C, and serum samples were stored at −80 °C until immunological analyses were performed. Sampled fish were then humanely killed through an overdose of anesthetic (0.5% clove oil) followed by cervical transection in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.6. Biochemical Analyses

2.6.1. Proximate Composition

The proximate compositional analysis of the diets and fish fillets (moisture, ash, protein and lipids) was determined using standard methods [53]. Moisture was quantified by the loss-on-drying method (LOD), in which pre-weighed samples placed in porcelain crucibles were dried at 105 °C for 24 h (method 950.46). Ash content was assessed through combustion in a muffle furnace for 12 h at 550 °C (method 920.153). Crude protein (N × 6.25, method 954.01) was determined using the Kjeldahl procedure after acid digestion (Kjeltec Auto Tecator, Foss A/S, Hillerød, Denmark) (method 988.05). Total lipid content in the diets was analyzed by first subjecting samples to acid hydrolysis with hydrochloric acid, followed by petroleum ether extraction in a Soxhlet apparatus (Soxtec System HT 1043 Extraction Unit, Foss A/S, Hillerød, Denmark) in accordance with ISO 6492:1999 for the determination of fat content in animal feeding stuff.

2.6.2. Determination of the Tissue Fatty Acid Profiles

The fatty acid composition of the experimental diets and fish fillets was evaluated following extraction and direct transesterification to fatty acid methyl esters (FAME) using a methanol-toluene (3:2) and an acetyl chloride-methanol solution 1:20 (v/v) (modified method [54]). In brief, FAME were analyzed through gas chromatography-mass spectrometry (7890A GC system, Agilent Technologies, Santa Clara, CA, USA), using an Agilent DB-WAX GC column (30 m 0.25 mm, 0.25 µm film thickness). The oven temperature program began at 130 °C and increased to 215 °C at 3 °C min^{-1}, followed by a gradual rise to 220 °C at 0.3 °C min^{-1}, and then to 240 °C at 20 °C min^{-1} where it was maintained for 12 min. The temperatures of the MS source and quadrupole were set to 230 °C and 150 °C, respectively. Analyses were performed using split injection mode. Fatty acid identification was achieved by comparing the obtained mass spectra with those of authentic standards and entries in the NIST mass spectral database (NIST/EPA/NIH library, Search Program 2.0f). Individual fatty acid concentrations were expressed as the percentage of the total lipids. Nutritional quality indices of the lipid fraction were calculated from the FA composition analysis of the fish fillets [55,56]. From the fatty acid profile, the Hypocholesterolaemic/hypercholesterolaemic ratio (h/H), the Atherogenic Index (AI), the Thrombogenic index (TI), the Unsaturation Index (UI), and the fish lipid quality (FLQ) were calculated using the following equations where Σ n-6 PUFA and Σ n-3 PUFA were the sum of omega 6 and omega 3 polyunsaturated fatty acids, Σ MUFA was the total of monounsaturated fatty acids and Σ SFA was the total saturated fatty acids:

$$h/H = (C18{:}1\ n\text{-}9 + \Sigma\ n\text{-}6\ PUFA + \Sigma\ n\text{-}3\ PUFA)/(C12{:}0 + C14{:}0 + C16{:}0)$$

$$AI = (C12{:}0 + 4 \times C14{:}0 + C16{:}0)/\Sigma\ \text{unsaturated FA},$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/[(0.5 \times \Sigma\ MUFA) + (0.5 \times \Sigma\ n\text{-}6\ PUFA) + (3 \times \Sigma\ n\text{-}3\ PUFA) + (\Sigma\ n\text{-}3\ PUFA/\Sigma\ n\text{-}6\ PUFA)],$$

$$UI = 1 \times (\Sigma\ \text{monoenoic FA}) + 2 \times (\Sigma\ \text{dienoic FA}) + 3 \times (\Sigma\ \text{trienoic FA}) + 4 \times (\Sigma\ \text{tetraenoic FA}) + 5 \times (\Sigma\ \text{pentaenoic FA}) + 6 \times (\Sigma\ \text{hexaenoic FA}),$$

$$FLQ = 100 \times (C22{:}6\ n\text{-}3 + C20{:}5\ n\text{-}3)/\Sigma\ SFA$$

2.7. Intestine and Liver Histology

Standard histological methods were used to prepare liver and intestine sections [57]. Briefly, tissues fixed in 10% buffered formalin were washed in 70% ethanol, dehydrated in

ascending methanol/ethanol concentrations to absolute concentration. Following dehydration, samples were embedded in paraffin wax at 60 °C. Thin sections (2–4 µm) were cut using a microtome (RM2255, Leica Biosystems, Nussloch, Germany), stained with hematoxylin and eosin using an automated staining system (Auto Stainer XL, Leica Biosystems, Nussloch, Germany) and examined under a light microscope (VANOX-T, Olympus, Center Valley, PA, USA) equipped with a digital camera (Infinity, Teledyne Lumenera, Ottawa, ON, Canada). Posterior intestine sections were evaluated for the presence of absorptive vacuoles within the mucosal enterocytes and for mucosal and submucosal integrity. Liver sections were evaluated for signs of lipid accumulation and the preservation of hepatocyte morphology. Representative micrographs were captured and analyzed using dedicated image analysis software (Image Pro Plus v6.0.0.260) according to Kokou and colleagues [58].

2.8. Hematological and Immunological Parameters

All hematological and immune parameters were assessed using a microplate spectrophotometer (Fluostar, BMG Labtech, Offenburg, Germany). Hemoglobin concentration was measured in the heparinized blood using the Drabkin method [59] with methemoglobin as the standard and miniaturized for assessment in 96-well microplates [60]. Immune parameters were assessed in 384-wells microplates in the serum samples stored at −80 °C. The serum antibacterial activities of lysozyme against *Micrococcus luteus* and complement against a luminescent strain of *Escherichia coli* were assessed as previously described [61]. The anti-protease activity was determined using azocasein as substrate and porcine pancreas trypsin as standard [62]. Serum nitric oxide was assessed using sodium nitrite as the standard through the Griess method [61]. Serum myeloperoxidase activity was determined using 2.5 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride and 5 mM hydrogen peroxide as substrate [60,63]. Ceruloplasmin activity was measured using p-phenylenediamine as substrate as described before [62]. The alkaline phosphatase activity was determined as described recently [64].

2.9. Experimental Design and Statistical Analysis

Regarding the experimental design (see graphical abstract), in the first (growth) phase, a balanced 3-block design was applied with 3 experimental tanks, each containing 3 nets randomly assigned to the 3 dietary treatments (FO, AO, and RO), while a 4th technical validation tank containing 3 nets all supplied with RO was used as a diagnostic control to evaluate potential between-tank and within-tank (net-to-net) variability. A two-way ANOVA analysis was thus performed only on fish fed the RO diet either in the 3-block design or in the validation tank to determine the effect of the net's position in the tank and of the interaction between nets and the tank. Then, one-way ANOVA was performed only on the 3-block design tanks (triplicate nets/diet) after the 13 wk growth trial to determine the effect of the diets on fish growth, proximate composition, and FA profile. For the second (finishing) phase, technical duplication (two tanks per treatment) was applied due to logistical constraints and the distinct objective of this phase; while the initial 13-week growth period, which examined the dose–response effects of dietary RO on fish growth, was conducted using triplicate tanks as standard practice, the finishing phase focused on fatty acid composition of the fillet and fish immune responses. To mitigate reduced tank replication, a higher number of fish per net was sampled to increase the number of biological replicates within dietary treatments, each treated as fixed factors in the ANOVA analysis as before. The larger sample size, rigorous analytical procedures, repeated measurements, and internal standards further ensured quality control and data reliability.

Prior to statistical analysis, proportional data (percentage and fatty acid profiles) were transformed using arcsine square root to meet ANOVA assumptions of normality and

homoscedasticity, checked using the Shapiro–Wilk and the Levene tests, respectively. When assumptions were met, data were analyzed using Analysis of Variance (ANOVA). Post hoc comparisons were performed using Tukey's HSD test when significant differences were detected ($\alpha = 0.05$). When assumptions were not met despite the transformations, non-parametric tests were performed, i.e., Kruskal–Wallis followed by the Games–Howell Post Hoc test. Hematological and immune parameters were analyzed using linear mixed-effects models, with diet included as a fixed factor and tank included as a random factor to account for the nested experimental design. When a significant dietary effect was detected, estimated marginal means were compared using Tukey-adjusted pairwise comparisons.

At the end of the growth phase, linear and quadratic effects of dietary rapeseed oil inclusion were assessed using polynomial contrast analysis based on dietary levels of RO inclusion in FO, AO, and RO diets (6%, 9%, and 12% respectively). These polynomial regression analyses were not applied at the end of the finishing phase because dietary lipid composition was no longer linearly graded across treatments due to the intermediate switching of diets. The simple contrast analysis was performed for the immunological parameters to compare each experimental dietary treatment to the FOFO group.

In addition, fatty acid datasets and lipid quality indices were further explored using multivariate approaches. Variables showing significant differences among diets were standardized (zero mean and unit variance) prior to Principal Component Analysis (PCA), which was used to reduce dimensionality and explore overall patterns among dietary groups. Separate PCA analyses were performed for fatty acid profiles and lipid quality indices, and results were visualized using 2 biplots to illustrate relationships between variables and diet clusters represented by ellipses.

All statistical tests were performed with a significance level of $\alpha = 0.05$ using JASP Statistical Software (v 0.95.4; JASP Team, 2025), except for PCA, which was performed and presented using R Studio (v 4.4.2; R Core Team, 2024) using the fviz_pca_biplot function.

3. Results

3.1. Fish Growth, Feed Utilization and Morphometric Indices

Comparing the fourth validation tank and the three RO nets in the 3-block design, consistent growth was obtained across independent rearing units (Table 4). No significant differences in fish weight were observed among fish fed the RO diet at any sampling point (t0, 56d and 93d), regardless of whether the fish were reared in nets positioned differently, in separate nets within the validation tank or in distinct tanks under the three block experimental design. These results indicate that neither the position of the net within the tank (net effect) nor the different tanks (tank effect) influenced fish weight during the experimental period (one-way ANOVA, p-value > 0.05).

Table 4. Statistical analysis of the weights of fish fed the RO diet in the 3-block randomized tanks or in the validation tank; effect of the net position in the large tanks (Net effet) and of the tank (nets in distinct tanks, i.e., 3-block tanks or nets in the same tank, i.e., validation tank).

One-Way ANOVA (*P*)	Net Effect	Tank Effect	Net × Tank Effect
Wi (n = 20)	0.997	0.554	0.713
W 56d (n = 20)	0.745	0.655	0.613
W 93d (13 wk, n = 12)	0.581	0.436	0.425

All fish, fed the RO diet for the 13 wk-growth phase, were distributed either in nets in the first, second or third position of the 3-block tanks, or in the 3 nets of the validation tank. Fish were weighed at the beginning of the trial (initial weight, Wi), at the first intermediate sampling after 56 days (W 56d) and at the end of the growth trial (W 93d = 13 wk). The number of fish in each net (n) varied from 20 to 12 fish/net from the start to the end of the growth trial.

Fish performance, feed utilization and morphometric results are presented in Table 5. Fish growth was slower during the 13 wk growth period than during the final 12 wk-finishing period (mean SGR 0.27 ± 0.01 and 0.47 ± 0.02 and mean FCR 2.05 ± 0.12 and 1.50 ± 0.05, respectively). At the end of the 13 wk-growth period, there were no significant differences in growth performance ($p > 0.05$) or feed utilization among the dietary groups. Identically, during the 12 wk-finishing period alone and when the entire growth period (0–25 wk) was considered, fish growth and feed utilization did not show any differences among the dietary groups ($p > 0.05$, Table 5). The three morphometric parameters tested at the end of the finishing period (25 wk) showed significant differences among the diets, with HSI significantly reduced in fish fed AOAO, ROFO and ROAO compared to fish fed RORO, whilst VSI and MFI were significantly reduced in fish fed ROAO compared to fish fed RORO ($p < 0.05$). VSI was also significantly reduced in fish fed ROFO compared to fish fed RORO or FOFO ($p = 0.001$). FO consumption was reduced by 70% in fish fed diets AOAO, RORO and ROAO and by 35% in fish fed ROFO compared to control fish fed FO throughout the trial.

Table 5. Effect of dietary oil on fish growth performance, feed utilization and morphometric indices of European sea bass fed 25 wk with the 3 different lipid sources (fish oil, FOFO; algal oil, AOAO; rapeseed oil, RORO) or for 13 wk with the RO diet followed by a 12-week finishing period with the FO or AO diets (ROFO and ROAO respectively).

	FOFO	**AOAO**	**RORO**	**ROFO**	**ROAO**	**SEM**	***p*-Values**		
							T	L	Q
Wi, g/fish [1]	200.22	193.38	195.10			2.597	0.565	0.423	0.433
Growth period (13 wk)									
BW, g/fish [1]	260.00	246.23	247.83			4.073	0.470	0.094	0.214
WG, % [1,4]	29.86	27.34	27.00			1.071	0.457	0.338	0.662
DFI, g/fish/day [1,5]	2.12	2.12	2.09			0.024	0.870	0.674	0.774
SGR, %/day [1,6]	0.28	0.26	0.26			0.009	0.453	0.337	0.680
FCR [1,7]	2.01	2.28	1.86			0.115	0.355	0.585	0.194
Finishing period (12 wk)									
Wf, g/fish [2]	393.66	365.10	357.22	375.71	355.93	7.081	0.505		
WG, % [2,4]	51.15	48.95	43.26	44.80	44.20	2.156	0.831		
DFI, g/fish/day [2,5]	2.24	2.13	2.12	2.16	2.04	0.040	0.726		
SGR, %/day [2,6]	0.51	0.49	0.45	0.46	0.46	0.018	0.821		
FCR [2,7]	1.51	1.48	1.55	1.44	1.51	0.018	0.399		
Total trial (25 wk)									
WG, % [2,4]	97.94	90.32	81.46	91.01	80.25	3.301	0.492		
DFI, g/fish/day [2,5]	4.34	4.20	4.17	4.35	4.11	0.059	0.526		
SGR, %/day [2,6]	0.39	0.37	0.34	0.37	0.34	0.010	0.493		
HSI, % [3,8]	1.70 ^{ab}	1.35 ^{a}	2.01 ^{b}	1.59 ^{a}	1.56 ^{a}	0.067	0.006		
VSI, % [3,9]	10.67 ^{c}	9.91 ^{bc}	10.60 ^{c}	9.66 ^{b}	8.57 ^{a}	0.267	0.001		
MFI, % [3,10]	4.71 ^{ab}	4.75 ^{ab}	5.20 ^{b}	4.44 ^{ab}	3.64 ^{a}	0.186	0.021		
Total FO consumption (g/net) [11]	303.28 ^{c}	93.22 ^{a}	93.75 ^{a}	197.34 ^{b}	91.66 ^{a}	28.25	0.018		

SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic; Wi = initial body weight; BW = body weight; Wf = final body weight; WG, percentage of weight gain; FI = feed intake; SGR = specific growth rate; FCR = feed conversion ratio; HSI = hepatosomatic index; VSI = viscerosomatic index; MFI = mesenteric fat index. $^{a–c}$ Different letters in the same row show significant differences between experimental diets (one-way ANOVA, $p < 0.05$). [1] The data are the means of 3 net replicates of 20 fish in each group ($n = 3$). [2] The data are the means of 2 net replicates of 9 fish in each group ($n = 2$). [3] The data are the means of 2 net replicates of 6 fish in each group ($n = 2$). The growth performance indices were calculated based on the initial body weight (Wi, g/fish), fish weight (Wt, g/fish) at sampling time t (days), and the biomass of each net at the start (Bi, g) and at sampling time t (Bt, g) as follows: [4] WG (Weight Gain, %) = 100 × (Wf − Wi) /Wi. [5] DFI (Daily Feed Intake, g/fish/day) = total feed intake/number of fish/feeding days. [6] SGR (Specific Growth Rate, %/day) = (Ln Wf − Ln Wi) × 100/feeding days. [7] FCR (Feed Conversion Ratio) = total feed intake/(Biomass t − Biomass i). [8] HSI (%) = 100 × (Weight liver/BW). [9] VSI (%) = 100 × (Weight viscera/BW). [10] MFI (%) = 100 × (Weight visceral fat/BW). [11] Total FO consumption (g/net) = Feed consumption during growth phase × % FO in diet + feed consumption during finishing phase × % FO in diet.

3.2. Proximate Composition of the Fish Fillet

The proximate composition of the fish fillets did not show any significant difference between the dietary treatments at either 13 or 25 wk (Table 6) except for the protein composition of the fillets at the end of the growth period which was significantly higher in fish fed AO or RO diets compared to fish fed the FO diet ($p = 0.011$) with a linear effect of dietary RO ($p = 0.010$). This was not apparent after 25 wk of the trial.

Table 6. Proximate composition (analyzed, not calculated) of the fillet of European sea bass after 13 or 25 wk of feeding the 3 different experimental diets (FOFO, AOAO, RORO) or after 12 wk of feeding the FO or AO finishing diets in fish fed the RO diet for the first 13 wk (ROFO, ROAO).

	FOFO	AOAO	RORO	ROFO	ROAO	SEM	*p*-Values		
	End of growth period (13 wk)						T	L	Q
Moisture [1]	76.58	76.57	75.94			0.135	0.079	0.050	0.244
Ash [1]	1.42	1.38	1.41			0.037	0.942	0.951	0.738
Proteins [1]	17.41 [a]	18.69 [b]	18.60 [b]			0.211	0.011	0.010	0.071
Lipids [1]	4.59	3.73	4.28			0.194	0.192	0.515	0.092
	End of the trial (25 wk)								
Moisture [2]	75.20	75.60	75.45	75.90	75.08	0.113	0.150		
Ash [2]	1.38	1.44	1.33	1.38	1.42	0.014	0.087		
Proteins [2]	19.30	20.08	18.81	19.37	19.72	0.170	0.294		
Lipids [2]	4.10	3.06	4.83	3.39	4.40	0.254	0.152		

SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic. [1] The data are the means of 3 replicates of 2 fish per replicate ($n = 6$). [2] The data are the means of 2 replicates of 3 fishes per replicate ($n = 6$). Different letters in the same row show significant differences between diets (one-way ANOVA, $p < 0.05$).

3.3. Fatty Acids of the Fish Fillet

The results of the FA profile of the fish fillets at the end of the growth and finishing periods are presented in Tables 7 and 8, respectively. The fatty acids profile of the sea bass fillet reflected the dietary fatty acid profile (Table 3). At the end of the 13 wk-growth trial, n-9 MUFA ($p = 0.007$), mainly the 18:1 n-9 (oleic acid, OA, $p = 0.009$), and h/H ($p = 0.001$) were significantly increased in fish fed the RO diet compared to the control FO group. These dose-response increases were linear in function of the dietary RO content ($p \leq 0.003$). On the contrary, SFA ($p < 0.001$), 16:1 n-7 ($p = 0.001$), n-3 PUFA ($p = 0.039$), mainly stearidonic acid (SDA, $p = 0.002$) and EPA ($p < 0.001$), n-3/n-6 ($p = 0.054$), AI ($p < 0.001$) and EPA+DHA ($p = 0.028$), were significantly lower than that of fish fed FO and decreased in a linear manner when dietary RO was considered ($p < 0.05$). In AO-fed fish, ARA (20:4 n-6) and h/H were highly significantly increased (both at $p = 0.001$), while SFA ($p < 0.001$), 16:1 n-9 ($p < 0.001$), SDA ($p = 0.002$), AI ($p < 0.001$) and TI ($p = 0.029$) were reduced compared to the FO-fed fish giving significant quadratic regressions in function of the dietary RO ($p < 0.05$).

Table 7. Effect of dietary oil on the fatty acid profile (% of total fatty acids) of the fillet of European sea bass after 13 wk feeding the 3 different experimental diets (FO, AO, RO).

	FO	AO	Δ	RO	Δ	SEM	*p*-Value		
							T	*L*	*Q*
14:0	2.40 [b]	1.98 [a]	(−17.5)	2.01 [a]	(−16.3)	0.057	<0.001	<0.001	0.007
16:0, PA	15.92 [b]	15.42 [ab]		15.07 [a]	(−5.3)	0.135	0.022	0.007	0.746
18:0, SA	4.30	4.15		4.20		0.045	0.416	0.367	0.335
Σ SFA [1]	23.88 [b]	22.75 [a]	(−4.7)	22.39 [a]	(−6.2)	0.194	<0.001	<0.001	0.173
16:1 n-7, POA	3.59 [b]	3.39 [b]		3.03 [a]	(−15.5)	0.073	0.001	<0.001	0.456
16:1 n-9, HDA	0.46 [b]	0.38 [a]	(−17.5)	0.41 [ab]		0.010	<0.001	0.024	0.001
18:1 n-7	2.87	2.83		2.86		0.016	0.654	0.937	0.367

Table 7. *Cont.*

	FO	AO	Δ	RO	Δ	SEM	*p*-Value		
18:1 n-9, OA	27.73 [a]	28.41 [ab]		30.12 [b]	(+8.6)	0.357	0.009	0.003	0.390
20:1 n-9	2.89	2.81		2.99		0.043	0.232	0.309	0.166
22:1 n-11, EA	1.83	1.70		1.73		0.048	0.541	0.436	0.436
24:1n-9	0.56	0.61		0.54		0.017	0.224	0.613	0.101
Σ MUFA [1]	41.20	41.29		42.89		0.338	0.063	0.036	0.252
Σ n-9 MUFA [1]	32.08 [a]	32.66 [a]		34.51 [b]	(+7.6)	0.361	0.007	0.003	0.295
18:2 n-6, LA	15.03	15.51		16.18		0.279	0.249	0.102	0.873
20:2 n-6, EDA	0.82	0.82		0.85		0.012	0.502	0.277	0.684
20:4 n-6, ARA	0.56 [a]	0.92 [b]	(+64)	0.65 [a]		0.049	0.001	0.280	<0.001
Σ n-6 PUFA [1]	16.76	17.67		18.09		0.282	0.146	0.059	0.666
18:3 n-3, ALA	2.96	3.12		3.27		0.055	0.066	0.022	0.931
18:4 n-3, SDA	0.68 [b]	0.56 [a]	(−18.4)	0.58 [a]	(−15.1)	0.017	0.002	0.003	0.013
20:5 n-3, EPA	3.86 [b]	3.59 [ab]		3.34 [a]	(−13.5)	0.066	<0.001	<0.001	0.922
22:5 n-3, DPA	0.87	0.77		0.78		0.022	0.105	0.088	0.186
22:6 n-3, DHA	9.13	9.43		7.99		0.268	0.060	0.069	0.105
Σ n-3 PUFA [1]	18.15 [ab]	18.28 [b]		16.64 [a]	(−8.3)	0.305	0.039	0.032	0.129
n-3/n-6 PUFA	1.09 [b]	1.04 [ab]		0.93 [a]	(−14.7)	0.029	0.054	0.020	0.556
h/H [2]	3.42 [a]	3.70 [b]	(+7.9)	3.80 [b]	(+12.7)	0.051	0.001	<0.001	0.233
AI [3]	0.24 [b]	0.21 [a]	(−10.2)	0.21 [a]	(−12.7)	0.004	<0.001	<0.001	0.034
TI [4]	0.27 [b]	0.25 [a]	(−5.6)	0.26 [ab]		0.002	0.029	0.266	0.014
UI [5]	168.62	171.77		163.89		1.401	0.059	0.138	0.053
FLQ [6]	54.40	57.19		50.61		1.213	0.076	0.175	0.060
EPA + DHA [7]	0.59 [b]	0.47 [ab]		0.42 [a]	(−29.1)	0.028	0.028	0.010	0.524

Diets FO = fish oil; AO = algal oil; RO = rapeseed oil; T = treatment; L = linear; Q = quadratic; AI = atherogenecity index; ALA = α-linolenic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EA = erucic acid; EDA = eicosadienoic acid; EPA = eicosapentaenoic acid; ERA = eicosatrienoic acid; FLQ = fish lipid quality; HDA = hypogenoic acid; h/H = hypocholesterolaemic/hypercholesterolaemic ratio; LA = linoleic acid; MUFA = monounsaturated fatty acids; n-3/n-6 PUFA = ratio of omega 3/omega 6 FA; OA = oleic acid; PA = palmitic acid; POA = palmitoleic acid; PUFA = polyunsaturated fatty acids; SA = stearic acid; SDA = stearidonic acid; SEM = standard error of the mean; SFA = saturated fatty acids; TI = thrombogenicity index; UI = Unsaturation Index; Σ = sum. Results are expressed as the means of 3 replicates of 2 fish per replicate (n = 6). [a–b] Different letters on the same line show significant differences in one type of FA or in a lipid nutritional quality index among dietary treatments (FO, AO or RO) of fish fed for 13 wk. Where a dietary group showed significant difference in comparison with fish fed the FO-based diet ($p < 0.05$), the percentage of change compared to FO-fed fish is given in brackets in the adjacent column (Δ, %). [1] Include fatty acids not listed (<0.5% of total FA). [2] h/H = Hypocholesterolaemic/hypercholesterolaemic ratio (h/H). [3] AI = Atherogenecity index. [4] TI = thrombogenicity index. [5] UI = Unsaturation Index. [6] FLQ = fish lipid quality. [7] EPA+DHA is expressed as g/100 g fillet.

At the end of the finishing trial (Table 8, 25 wk), the differences in FA profile became significant for all FA groups. A significant decrease in SFA ($p < 0.001$), and n-3 PUFA (20:5 n-3, EPA, $p < 0.001$; 22:5 n-3, docosapentaenoic acid, DPA, $p = 0.002$; 22:6 n-3, DHA, $p < 0.001$) and a highly significant increase in oleic acid (18:1 n-9, OA, $p < 0.001$) and linoleic acid (18:2 n-6, LA, $p < 0.001$) were observed in the group of fish reared on the RORO diet compared to the FOFO diet. There was a significant decrease in the n-3/n-6 PUFA ratio ($p < 0.001$) in RORO-fed fish compared to FOFO-fed fish, which was fully recovered in fish fed ROFO but only partially in fish fed ROAO. Significant reductions in n-3 PUFA levels, particularly in EPA, DHA, and their intermediate DPA, were observed in RORO-fed fish, with decreases of 31%, 38%, and 20.7%, respectively ($p \leq 0.002$) compared to FO-fed fish resulting in a 25.5% reduction of total n-3 PUFA levels ($p < 0.001$). In addition, AOAO-fed fish also showed highly significant reduction in EPA (−11.9%, $p < 0.001$) and its precursor stearidonic acid (SDA, 18:4 n-3, −24.5%, $p = 0.002$) but not in DPA or DHA levels compared to FOFO-fed fish. Almost complete recovery (96.6%) of the total n-3 PUFA levels was suggested in fish fed ROFO while partial but still high recovery (88.4%) was evident for diet ROAO. The plant oil diet resulted in lower AI, UI and FLQ ($p < 0.001$) and higher h/H

($p < 0.001$) compared to the FO and AO diets. UI and FLQ returned to the levels of FO-fed fish in fish switched to FO or AO for 12 wk. Concerning h/H and AI, changes observed in RORO fish were halved in fish switched to the FO diet, and somewhat less resolved in fish fed AO for the last 12 wk.

Table 8. Effect of dietary oil on the fatty acid profile (% of total fatty acids) of the fillet of European sea bass after 25 wk feeding the 3 different experimental diets (FOFO, AOAO, RORO) or after 12 wk of FO or AO finishing diets in fish fed the RO diet for the first 13 wk (ROFO, ROAO).

	FOFO	AOAO	Δ	RORO	Δ	ROFO	Δ	ROAO	Δ	SEM	*p*-Value
14:0	2.33 ^{c}	1.70 ^{a}	(−26.9)	1.66 ^{a}	(−28.6)	2.02 ^{b}	(−13.2)	1.79 ^{ab}	(−23.3)	0.063	<0.001
16:0, PA	15.71 ^{c}	14.90 ^{b}	(−5.2)	13.93 ^{a}	(−11.3)	14.82 ^{b}	(−5.6)	14.84 ^{b}	(−5.5)	0.143	<0.001
18:0, SA	4.40	4.13		4.01		4.28		4.04		0.052	0.078
Σ SFA [1]	23.89 ^{c}	21.94 ^{b}	(−8.2)	20.75 ^{a}	(−13.1)	22.49 ^{b}	(−5.9)	21.84 ^{b}	(−8.6)	0.247	<0.001
16:1 n-7, POA	3.45 ^{c}	3.30 ^{bc}		2.60 ^{a}	(−24.6)	3.01 ^{b}	(−12.7)	3.39 ^{bc}		0.080	<0.001
16:1 n-9, HDA	0.54 ^{c}	0.42 ^{a}	(−22.8)	0.49 ^{bc}		0.52 ^{c}		0.45 ^{ab}	(−15.7)	0.012	<0.001
18:1 n-7	2.81	2.70		2.80		2.77		2.77		0.027	0.738
18:1 n-9, OA	28.78 ^{a}	29.39 ^{ab}		34.40 ^{c}	(+19.5)	30.11 ^{ab}		30.64 ^{b}	(+6.5)	0.478	<0.001
20:1 n-9	2.20 ^{a}	2.41 ^{ab}		2.48 ^{ab}		2.24 ^{ab}		2.50 ^{b}	(+13.7)	0.039	0.013
22:1 n-11, EA	0.87	1.07		1.04		0.87		1.07		0.039	0.218
Σ MUFA [1]	40.08 ^{a}	40.66 ^{ab}		45.18 ^{c}	(+2.7)	40.96 ^{ab}		42.22 ^{b}	(+5.3)	0.447	<0.001
Σ n-9 MUFA	32.33 ^{a}	33.03 ^{ab}		38.16 ^{c}	(+8.0)	33.71 ^{ab}		34.37 ^{b}	(+6.3)	0.492	<0.001
18:2 n-6, LA	13.22 ^{a}	15.10 ^{b}	(+14.3)	16.78 ^{c}	(+26.9)	14.35 ^{ab}		15.34 ^{b}	(+16.0)	0.291	<0.001
20:2 n-6, EDA	0.72	0.80		0.73		0.82		0.79		0.014	0.102
20:4 n-6, ARA	0.68 ^{ab}	0.99 ^{b}	(+45.6)	0.46 ^{a}		0.66 ^{ab}		0.82 ^{b}		0.050	<0.001
Σ n-6 PUFA	14.96 ^{a}	17.28 ^{bc}	(+5.5)	18.39 ^{c}	(+22.9)	16.21 ^{b}	(+8.4)	17.33 ^{bc}	(+5.8)	0.288	<0.001
18:3 n-3, ALA	2.84 ^{a}	3.19 ^{b}	(+12.3)	3.75 ^{c}	(+32.0)	3.16 ^{b}	(+11.3)	3.32 ^{b}	(+16.9)	0.071	<0.001
18:4 n-3, SDA	0.57 ^{b}	0.43 ^{a}	(−24.5)	0.48 ^{ab}		0.53 ^{b}		0.48 ^{b}		0.013	0.002
20:5 n-3, EPA	4.15 ^{d}	3.68 ^{bc}	(−11.9)	2.86 ^{a}	(−31.0)	3.85 ^{c}	(−7.1)	3.54 ^{b}	(−16.7)	0.102	<0.001
22:5 n-3, DPA	0.76 ^{c}	0.67 ^{abc}		0.60 ^{a}	(−20.7)	0.73 ^{bc}		0.65 ^{ab}	(−14.0)	0.016	0.002
22:6 n-3, DHA	12.13 ^{c}	11.55 ^{bc}		7.50 ^{a}	(−38.0)	11.47 ^{bc}		10.06 ^{b}	(−16.5)	0.416	<0.001
Σ n-3 PUFA	21.06 ^{c}	20.12 ^{bc}		15.69 ^{a}	(−25.5)	20.34 ^{bc}		18.61 ^{b}	(−11.6)	0.473	<0.001
n-3/n-6 PUFA	1.40 ^{c}	1.17 ^{b}	(−14.3)	0.85 ^{a}	(−35.7)	1.26 ^{bc}		1.08 ^{b}	(−21.4)	0.046	<0.001
h/H [2]	3.59 ^{a}	4.02 ^{b}	(+11.9)	4.39 ^{c}	(+22.2)	3.96 ^{b}	(+10.2)	4.00 ^{b}	(+11.4)	0.062	<0.001
AI [3]	0.23 ^{c}	0.20 ^{b}	(−15.2)	0.18 ^{a}	(−23.8)	0.21 ^{b}	(−10.8)	0.20 ^{b}	(−15.2)	0.004	<0.001
TI [4]	0.24	0.23		0.25		0.23		0.24		0.002	0.080
UI [5]	182.1 ^{b}	182.1 ^{b}		160.6 ^{a}	(−11.7)	180.6 ^{b}		174.1 ^{b}		2.089	<0.001
FLQ [6]	68.19 ^{b}	69.42 ^{b}		50.01 ^{a}	(−26.7)	68.15 ^{b}		62.27 ^{b}		1.874	<0.001
EPA + DHA [7]	0.67	0.46		0.49		0.52		0.59		0.028	0.119

AI = atherogenecity index; ALA = α-linolenic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EA = Erucic acid; EDA = eicosadienoic acid; EPA = eicosapentaenoic acid; ERA = eicosatrienoic acid; FLQ = fish lipid quality; HDA = hypogenoic acid; h/H = hypocholesterolaemic/hypercholesterolaemic ratio; LA = linoleic acid; MUFA = monounsaturated fatty acids; n-3/n-6 PUFA = ratio of omega 3/omega 6 FA; OA = oleic acid; PA = palmitic acid; POA = palmitoleic acid; PUFA = polyunsaturated fatty acids; SA = stearic acid; SDA = stearidonic acid; SEM = standard error of the mean; SFA = saturated fatty acids; TI = thrombogenicity index; UI = Unsaturation Index; Σ = sum. Results are expressed as the mean ± S.D. The data are the means of 2 replicates of 3 fish per replicate ($n = 6$). $^{a–d}$ Different letters on the same line show significant differences between fish fed 25 wk with the 5 different dietary combinations (one-way ANOVA, $p < 0.05$). Where a dietary group showed significant difference in one type of FA or in a lipid nutritional quality index in comparison with fish fed the FOFO-based diet, the percentage of change compared to FOFO-fed fish is given in brackets in the adjacent column (Δ, %). [1] Include fatty acids not listed (<0.5% of total FA). [2] h/H = (C18:1 n-9 + Σ n-6 PUFA + Σ n-3 PUFA)/(C12:0 + C14:0 + C16:0). [3] AI = (C12:0 + 4 × C14:0 + C16:0)/Σ unsaturated FA. [4] TI = (C14:0 + C16:0 + C18:0)/[(0.5 × Σ MUFA) + (0.5 × Σ n-6 PUFA) + (3 × Σ n-3 PUFA) + (Σ n-3 PUFA/Σ n-6 PUFA)]. [5] UI = 1 × (Σ monoenoic FA) + 2 × (Σ dienoic FA) + 3 × (Σ trienoic FA) + 4 × (Σ tetraenoic FA) + 5 × (Σ pentaenoic FA) + 6 × (Σ hexaenoic FA). [6] FLQ = 100 × (C22:6 n-3 + C20:5 n-3)/Σ SFA. [7] EPA+DHA is expressed as g/100 g fillet.

The FA metabolism pathway can be visualized in Figure 1.

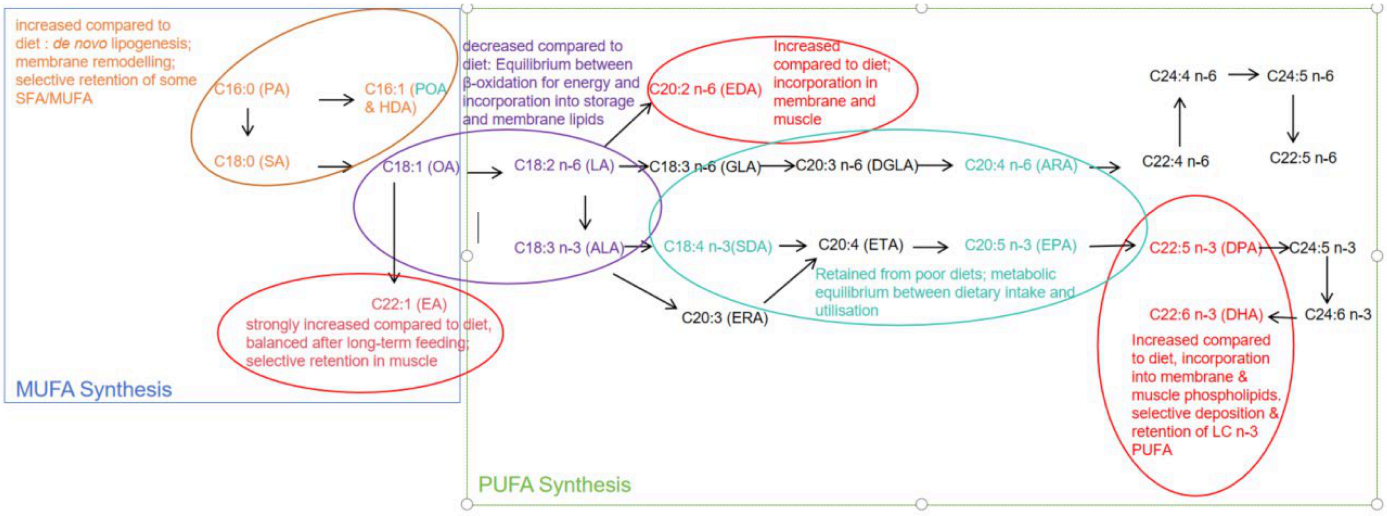

Figure 1. Biosynthetic pathway of polyunsaturated fatty acid with putative explanation of variations between dietary and muscle fatty acids (adapted from [17]).

As revealed by the Principal Component Analysis (PCA) presented in Figure 2A, the FA accounting for 77.8% of the differences in the FA profiles of the fish fed the experimental diets were mainly LA (18:2 n-6), ALA (18:3 n-3), OA (18:1 n-9), EPA (20:5 n-3) and DHA (22:6 n-3) on the horizontal axis, while levels of hypogeic acid (16:1 n-9), SDA (18:4 n-3) and ARA (20:4 n-6) distinguished the fish on the vertical axis. The quality indices of FA (Figure 2B) explained 95.9% of the differences between dietary treatments.

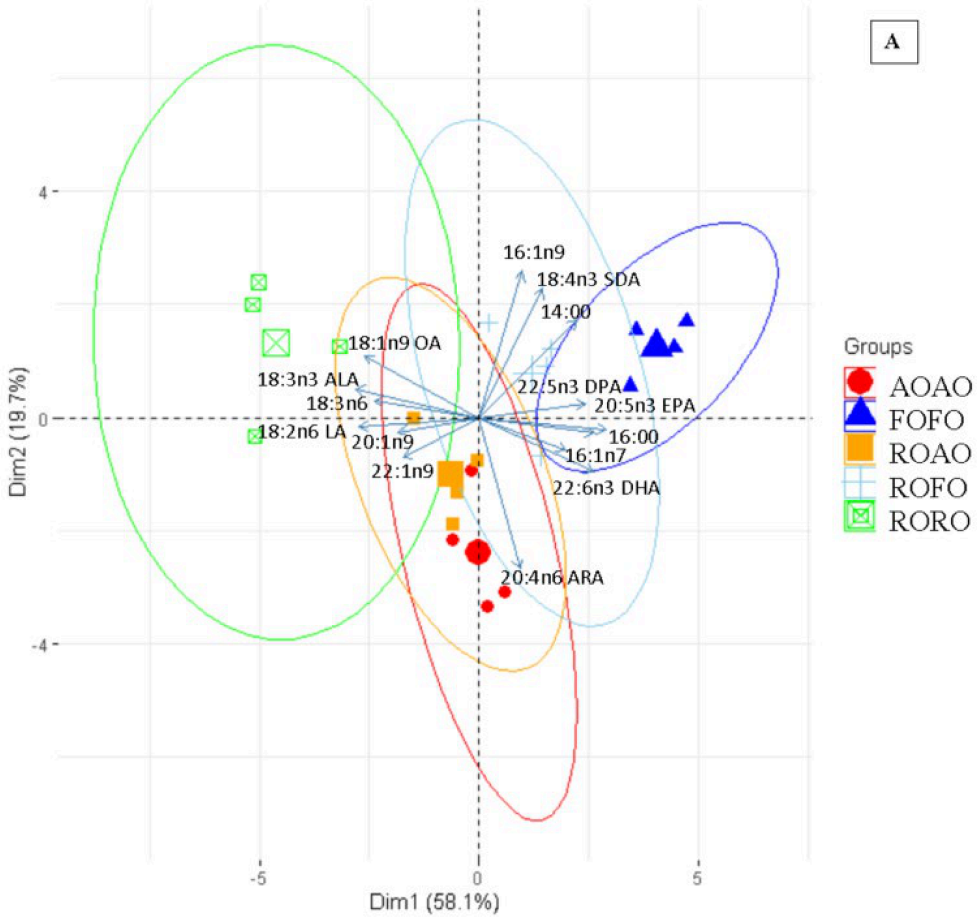

Figure 2. *Cont.*

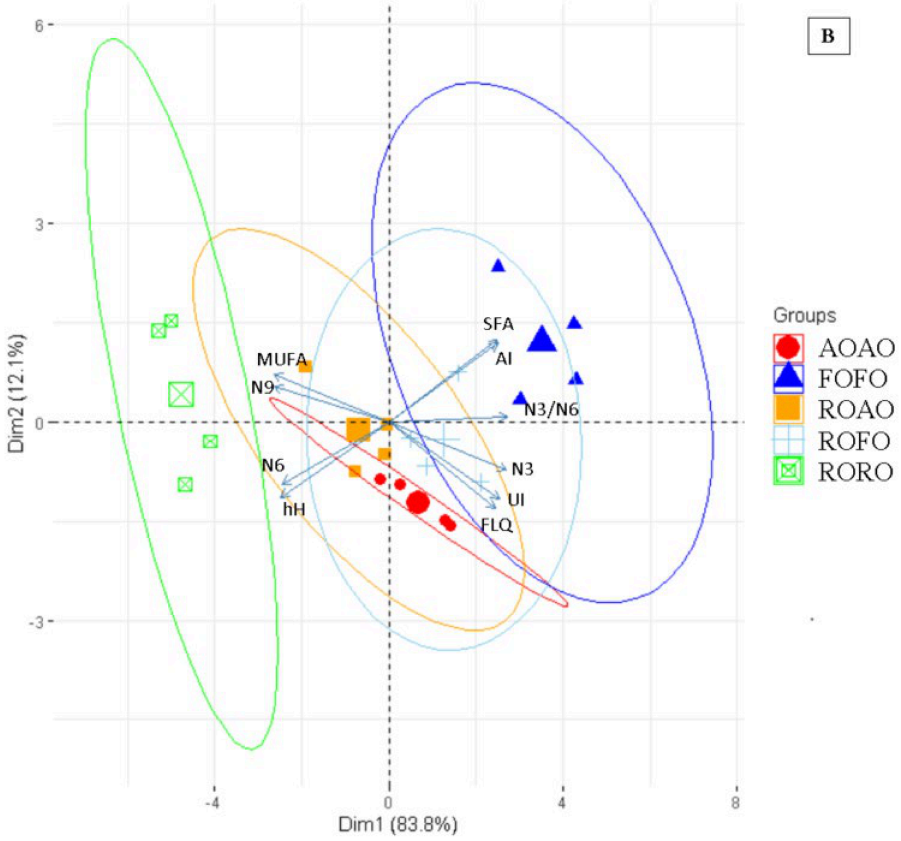

Figure 2. Principal Component Analysis biplots with (**A**) all FA significantly different between dietary treatments and (**B**) with all quality indices of fish fillet of European sea bass after 25 wk feeding with the 3 different experimental diets (FOFO, AOAO, RORO) or after 12 wk of finishing with the FO or AO diets in fish fed the RO diet for the first 13 wk (ROFO, ROAO). FO = fish oil; AO = algal oil; RO = rapeseed oil; Σ = sum; SFA = saturated fatty acids; OA = oleic acid; MUFA = monounsaturated fatty acids; LA = linoleic acid; ARA = arachidonic acid; PUFA = polyunsaturated fatty acids; ALA = α-linolenic acid; SDA = stearidonic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; h/H = hypocholesterolaemic/hypercholesterolaemic ratio (C18:1 n-9 + Σ n-6 PUFA + Σ n-3 PUFA)/(C12:0 + C14:0 + C16:0); AI = atherogenecity index (C12:0 + 4 × C14:0 + C16:0)/Σ unsaturated FA; TI = thrombogenicity index (C14:0 + C16:0 + C18:0)/[(0.5 × Σ MUFA) + (0.5 × Σ n-6 PUFA) + (3 × Σ n-3 PUFA) + (Σ n-3 PUFA/Σ n-6 PUFA)]; UI = Unsaturation Index = 1 × (Σ monoenoic FA) + 2 × (Σ dienoic FA) + 3 × (Σ trienoic FA) + 4 × (Σ tetraenoic FA) + 5 × (Σ pentaenoic FA) + 6 × (Σ hexaenoic FA); FLQ = fish lipid quality = 100 × (C22:6 n-3 + C20:5 n-3)/Σ SFA.

3.4. Histology

3.4.1. Liver Histology

At the end of the growth period (13 wk), cytoplasmic vacuolization of hepatocytes, consistent with lipid accumulation, was observed in liver sections from all dietary groups (Figure 3A–C). Qualitative examination of three different areas from each liver section revealed inter-individual variability. Specifically, three of the nine livers (33.3%) from the AO group (Figure 3B) appeared to exhibit less hepatocyte vacuolization than those from the FO control group (Figure 3A), whereas three of the nine livers (33.3%) from the RO group (Figure 3C) appeared to show more extensive lipid accumulation than the FO control group. Bile ducts and pancreatic tissue displayed normal histological features in all groups, with no evidence of inflammation. At the end of the finishing period (25 wk), qualitatively assessment suggested a slight increase in hepatocyte vacuolization in all dietary groups compared with the 13 wk sample (Figure 4A–C vs. Figure 3A–C). This increase appeared to be most pronounced in fish fed the RO diet throughout the trial. In contrast, fish switched

from the RO-based diet to either FO or AO finishing diets after 13 wk (Figure 4D,E) showed areas with reduced vacuolization in the liver sections, resulting in a heterogenous ("mosaic-like") appearance rather than homogenous vacuolization throughout the liver section of the RORO-fed fish (Figure 4D,E). This feature was particularly evident in fish reared on the AO finishing diet (Figure 4E). Variability among sections of each dietary group did not enable quantitative assessment, and these observations should be regarded as descriptive only.

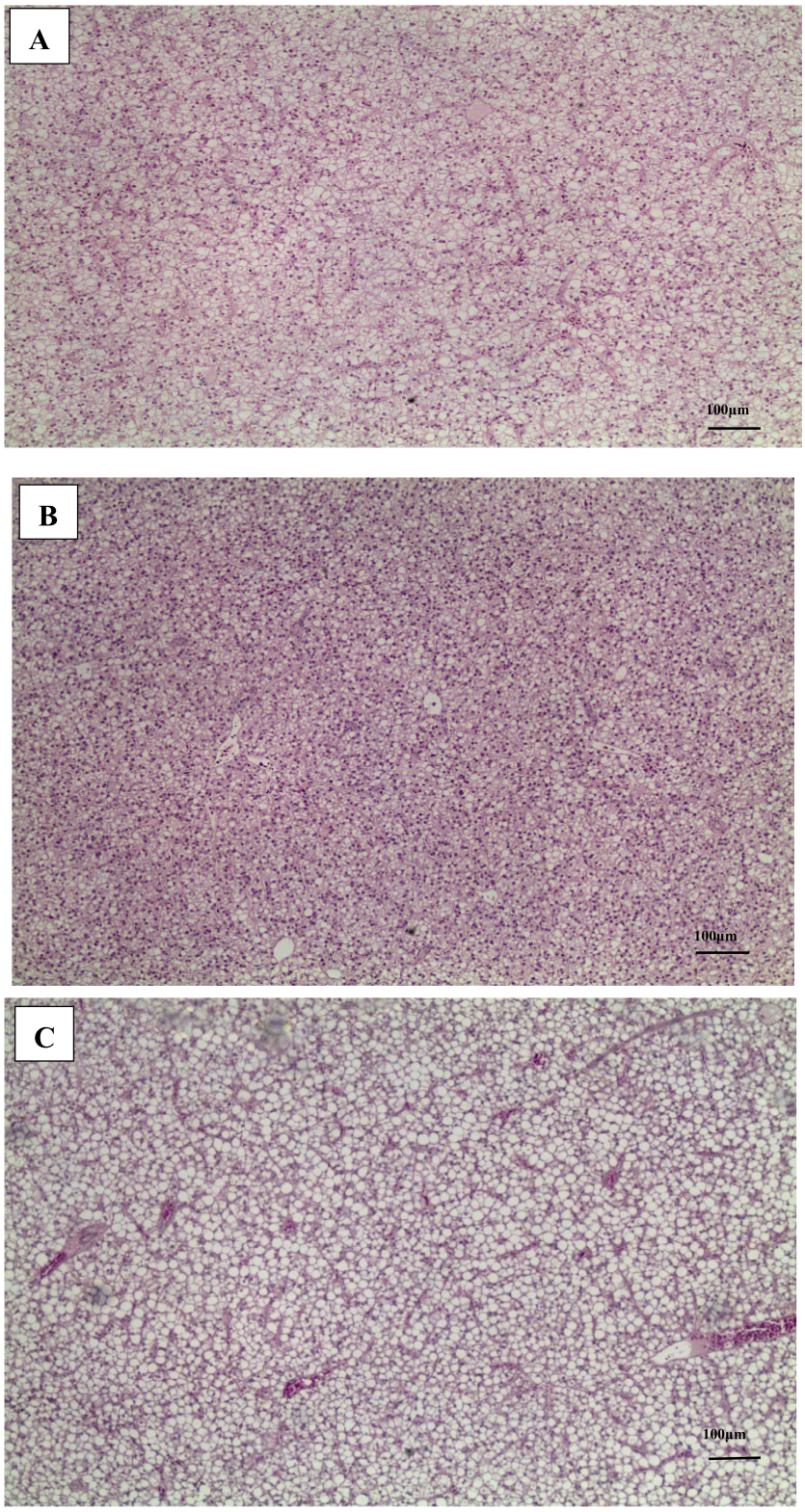

Figure 3. Liver sections at 13 wk of fish fed (**A**) FO diet, (**B**) AO diet and (**C**) RO diet (10×). $n = 9$.

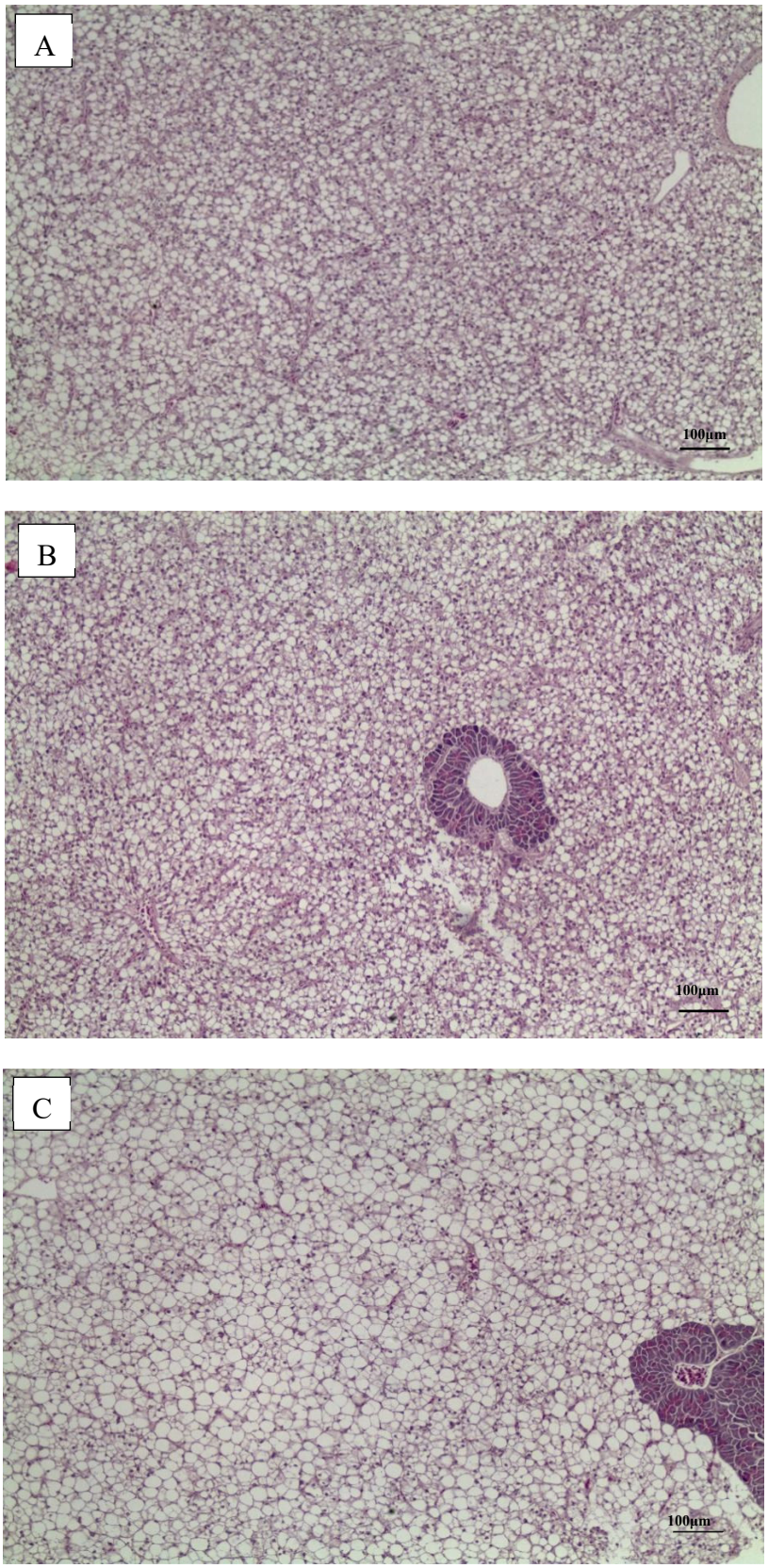

Figure 4. *Cont*.

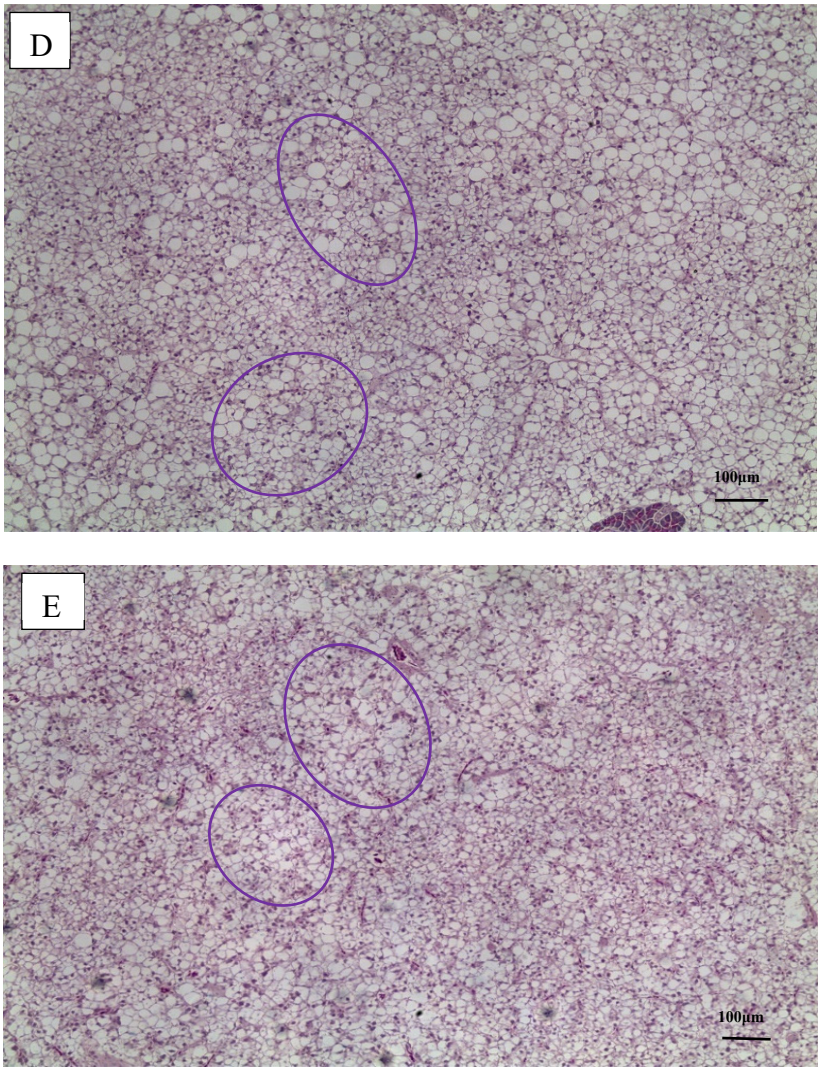

Figure 4. Liver sections of fish fed continuously (**A**) FO diet (FOFO), (**B**) AO diet (AOAO), (**C**) RO diet (RORO) for 25 wk, or fish fed RO for 13 wk and switched to (**D**) FO diet (ROFO) or (**E**) AO diet (ROAO) for the last 12 wk of the trial (10×). The ellipses show the mosaic-like features. n = 6.

3.4.2. Intestine Histology

After 13 wk of feeding the diets based on the different lipid sources, qualitative histological examination of the distal intestine revealed no apparent morphological differences among the dietary groups (Supplementary Figure S1). The intestinal epithelium appeared intact in six to nine of the nine fish examined per dietary treatment (66.7–100%), with no evidence of epithelial hypertrophy or hyperplasia. Enterocyte nuclei were located at the basal or mid-cell position and associated with supranuclear vacuoles of normal morphology. Eosinophilic granulocytes infiltration was observed in the intestinal mucosa of fish from all dietary groups (Figure 5). Although RO-fed fish appeared to exhibit a slightly increased abundance of these cells than fish fed the FO and AO diets, this observation was based solely on qualitative examination and not supported by cell counts. At the end of the finishing period (25 wk), no obvious qualitative differences in intestinal morphology were observed among dietary treatments (Supplementary Figure S2). As no quantitative histological assessment or statistical analysis was performed, these observations should be considered descriptive only.

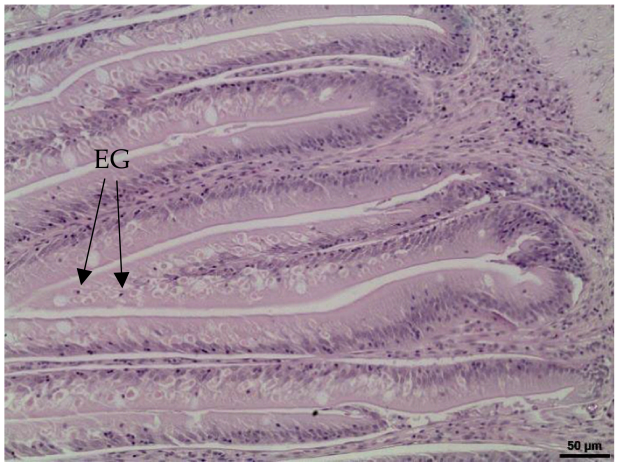

Figure 5. Posterior intestine sections (20×) showing eosinophilic granulocyte infiltration (EG). $n = 9$.

3.5. Haematological and Immunological Parameters

Regarding the haematology and immunology parameters at the end of the trial, no significant difference was found among the dietary treatments tested ($p > 0.05$, Table 9). Hemoglobin concentration was not affected by dietary lipids ($p = 0.934$). On the one hand, lysozyme activity, which measures antibacterial activity against a Gram-positive bacterium, *M. luteus*, tended to be increased in fish fed the RO-based diet throughout the trial compared to fish fed the AO-based diet either throughout the trial or as a finishing diet ($p = 0.090$). On the other hand, the antibacterial activity of the fish sera against a Gram-negative bacterium (*E. coli*) tended to be increased in fish fed the AO-based diet for the entire trial compared to fish fed the FO-based diet ($p = 0.050$). This antibacterial activity was significantly increased in AOAO-fed ($p < 0.001$), RORO-fed ($p = 0.007$) and ROAO-fed ($p = 0.002$) fish compared to FOFO-fed fish when simple contrast analysis was performed. The myeloperoxidase activity of serum showed no significant difference between dietary treatments ($p = 0.860$). Ceruloplasmin activity showed no significant differences between diets ($p = 0.513$), although fish response was elevated in all diets compared to fish fed FOFO and slightly less so in fish switched to FO or AO after 13 wk of RO feeding, but high inter-individual variation somewhat masked this effect. Alkaline phosphatase response followed the same pattern ($p = 0.554$).

Table 9. Hematological and serum immunological parameters at the end of the finishing period (at 25 wk) in European sea bass fed 13 wk with the 3 different lipid sources (FO, AO, RO) and then for another 12 wk with either the same diet as before (FOFO, AOAO, RORO) or with the FO or AO finishing diets in fish fed RO for the first 13 wk (ROFO and ROAO, respectively).

	FOFO	AOAO	RORO	ROFO	ROAO	SEM	*p*-Value
Hemoglobin, g/dL	14.17	15.49	16.33	15.01	16.64	0.817	0.934
Lysozyme, U/mL	600.93	526.47	654.38	582.67	543.61	17.393	0.090
E. coli growth inhibition, %	43.13	50.68 #	48.56 #	43.01	49.61 #	1.216	0.050
E. coli killing capacity, %	84.26	88.25	84.82	82.31	84.87	0.800	0.154
Myeloperoxidase activity, OD	0.17	0.11	0.16	0.15	0.15	0.014	0.860
Ceruloplasmin activity, U/mL	155.09	330.49	387.88	323.78	273.97	39.957	0.513
Alkaline phosphatase, U/mL	1.41	1.43	1.59	1.49	1.50	0.033	0.554

SEM = standard error of the mean. Results are represented as the means of 2 replicates of 8 fishes per replicate. No significant differences were present in any of the immune parameters (linear mixed model, nested ANOVA, $p > 0.05$). # Hash signs show significant difference between the tested diet and FOFO (simple contrast analysis, $p < 0.05$). ($n = 2$).

4. Discussion

Overfishing and climate change reduce the availability of FM and FO, driving the use of more sustainable aquafeed ingredients [65]. This study is the first long-term nutritional trial in European sea bass reared to market size using low-FM (17%) diets with RO, evaluating both a conventional FO finishing diet, and a finishing diet supplemented with EPA- and DHA-rich microalgae meals. The experimental diets replaced 66.7% of FO with RO alone or with RO and AO while maintaining balanced lipid levels. Unlike previous studies, which used higher FM diets (38–40%) [27,28,66] or limited FO replacement [50–52], this experimental design directly addresses the declining availability of marine ingredients by testing both partial FO replacement with algal oil and a microalgae-based finishing diet after prolonged feeding with plant oils.

4.1. Fish Growth and Morphometrics

In the present study, fish growth was slower during the first 13 wk than during the final 12 wk-finishing period (lower SGR and higher FCR), likely due to environmental stress caused by strong winds. Replacing 60–80% of FO with RO did not significantly affect fish growth or feed utilization after 13 or 25 weeks. Although fish fed the RORO diet showed a tendency toward higher FCR (1.55) than the FOFO control fish (1.51), it was improved (reduced) in fish fed ROFO (1.44) and ROAO (1.51), highlighting the effectiveness of the finishing strategy. Previous studies in European sea bass have reported variable effects of partial or total replacement of FO by plant oils on growth and feed efficiency depending on fish size and diet composition [9,27,28,66–68]. Likewise, dietary microalgae have shown species dependent effects, reducing growth in Atlantic salmon, *Salmo salar*, at high inclusion levels [69,70] but improving or not affecting growth in Nile tilapia, *Oreochromis niloticus*, turbot, *Scophthalmus maximus*, gilthead seabream, and European sea bass at lower inclusion levels [52,71–74]. In the present study, fish fed AO tended to show a better FCR (1.48) than fish fed FO (1.51) throughout the trial, although differences were not significant, indicating that the basal FO inclusion in all diets was sufficient to support optimal fish growth. Moreover, the finishing strategy significantly reduced FO consumption, by 70% in fish fed ROAO and by 35% in fish fed ROFO, compared to control fish fed FOFO.

In general, whole-body lipid content is proportional to dietary lipid levels, and excess dietary lipid is deposited in the liver, viscera, muscle, and adipose tissue of fish [75,76] thus making indices such as hepatosomatic and viscerosomatic indices good indicators of fat deposition. At the end of the trial, after 25 wk of feeding, hepatosomatic indices (HSI) were significantly decreased in AOAO-, ROFO- and ROAO-fed fish compared to RORO-fed fish, indicating a lower fat deposition in AO-fed fish and in fish fed both finishing diets suggesting that the finishing strategy may successfully mitigate the negative effects of long-term RO feeding. The significant increase in HSI in RO-fed fish in the present study was previously described in sea bass [66] and in RO-fed gilthead seabream [12]. Regarding VSI, it was not affected by 25 wk of RO feeding (RORO) but was significantly reduced in *D. labrax* fed RO for 13 wk followed by an FO- or AO-finishing diet for 12 wk (ROFO and ROAO respectively) compared to fish fed FO or RO throughout the trial (FOFO and RORO). Similarly, VSI was not affected in gilthead seabream [12] or in red hybrid tilapia, *Oreochromis* sp. [29] fed RO. Likewise, MFI was not significantly affected by dietary oil in these two fish species [12,29] or in European sea bass in the present study but tended to be decreased in fish fed ROFO compared with fish fed RORO and significantly so in fish fed ROAO. The reduced VSI and MFI, significantly or not, in fish switched to finishing diets compared to fish fed continuously with the same diet may suggest that the energy consumed to adapt to the dietary change may reduce the fat accumulation in and around their viscera.

4.2. Fish Fillet Proximate Composition

No significant differences were found in any of the nutrients analyzed in the fish fillets of the different dietary treatments except for the protein content of fish fillet at the end of the growth period, which was higher in fish fed AO or RO compared to fish fed FO. After long-term feeding (25 wk), this difference was not significant anymore. However, the lipid accumulation in the liver of RORO-fed fish suggested previously by the increased HSI was further evoked by a tendency towards increased lipids in their fillet compared to FOFO- and AOAO-fed fish after long-term feeding (25 wk). The finishing strategy with AO and especially with FO reduced this slight lipid accumulation observed in RORO-fed fish in the fillet. The lipid accumulation in the liver and fish fillet of RO-fed fish was also reversed by a finishing FO diet in rainbow trout [40].

4.3. Fish Fillet Fatty Acid Profile and Quality Indices

The present study on the partial replacement of dietary FO was based on three diets formulated to cover the basic FA requirements of European sea bass. This carnivorous marine fish has an extremely low/inexistent ability, compared to salmonids, to convert C18 precursors into long-chain PUFA and therefore has specific requirements for PUFA in its diet [8,14,17,18]. Diets were therefore formulated to include at least 3% of FO to cover the requirements of these FA for normal growth and development of marine fish [77]. Moreover, RO, rich in n-9 MUFA [20], was chosen over soybean oil, rich in n-6 FA, in order to avoid undesirable effects on the n-3/n-6 ratio [25].

In the present study, the fatty acid composition of the fish fillets was closely related to the dietary fatty acid input for most FA. As expected from the RO content in diets FO, AO, and RO at 6, 9, and 12%, respectively, a linear effect of dietary RO was seen on FA in the RO diet, while a quadratic effect was seen on FA in the AO diet. However, small differences in FA of interest between dietary treatments after 13 wk feeding became significant after 25 wk of feeding and suggest that tissue fatty acid profile does not in fact mirror dietary composition in a proportional manner. Instead, a hierarchical pattern of fatty acid retention and utilization was evident and can be visualized in Figure 1 and Supplementary Figure S3. Discrepancies between the dietary FA profile and the FA composition of fish fillets suggest that intake of oleic acid (OA, 18:1 n-9), linoleic acid (LA, 18:2 n-6), and α-linolenic acid (ALA, 18:3 n-3), along with selective deposition of specific FA, such as palmitic acid (PA, 16:0), stearic acid (SA, 18:0), palmitoleic acid (POA, 16:1 n-7), hypogenoic acid (HDA, 16:1 n-9), erucic acid (EA, 22:1 n-11), eicosadienoic acid (EDA, 20:2 n-6), docosapentaenoic acid (DPA, 22:5, n-3), and docosahexaenoic acid (DHA,22:6 n-3). Indeed, the levels of OA in fish fed the RO diet for 25 wk was higher (34.40%) compared to fish fed theFO diet (28.78%), but not as much as expected from the percentage of dietary OA (43.34% in diet RO, 31.60% in diet FO), suggesting utilization of this FA for energy purposes. Similar to the findings of Mourente and colleagues [28], the tendency toward increased levels of MUFA ($p = 0.063$) and n-6 PUFA ($p = 0.146$) (mainly OA and LA, respectively) in fish fed the RO diet for 13 wk compared to FO-fed fish became highly significant after 25 wk of feeding ($p < 0.001$) in the present study. Diets AO and especially RO, typically rich in LA and ALA, were only translated into significant increases in these PUFA in fish fillets after 25 wk (not after 13 wk), but not as strongly as dietary levels would have implied. In the present study, LA exhibited greater proportional incorporation into tissue lipids than ALA, suggesting moderate deposition with limited selective conservation of LA, while ALA consistently showing low retention relative to dietary supply and was preferentially utilized through β-oxidation rather than deposited in muscles [28]. The modest increase in tissue LA and ALA from 13 to 25 wk of feeding suggested that the tissue may be approaching a regulated equilibrium where additional ALA is increasingly diverted to metabolism

rather than deposition in muscle lipids. Fish fed the low-ARA RO diet initially maintained elevated tissue ARA levels, indicating selective conservation and likely recycling from membrane phospholipid pools, as ARA is an indispensable precursor of eicosanoids [78]. Over prolonged feeding, however, tissue ARA partially declined toward a lower steady state, suggesting dietary deficiency. This temporal pattern indicating an initial phase of rapid incorporation followed by a slower equilibration phase between 13 and 25 wk was also noticed in the case of PA, SDA, and EA.

Concerning DHA, levels in the fillets of RO-fed fish were much higher than dietary levels (almost double), suggesting that this PUFA is not only strongly conserved and selectively retained; it is maintained above dietary concentrations and protected from oxidation. This was also true of the fish fed the diets richer in DHA (FO and AO) and underlines that muscle lipid composition is physiologically regulated rather than simply mirroring diet. The fact that DHA was strongly conserved across diets and feeding durations, often exceeding dietary levels or remaining within a narrow physiological range, is probably due to its role as a structural membrane lipid. Selective deposition of DHA was also observed in other studies in European sea bass [27,67], gilthead seabream [12], and many other fish species [20,28,29,31,79,80] and reviewed recently by Xu and colleagues [81]. Fish are net producers of DHA, whether through de novo synthesis in low-trophic species or selective deposition in marine or high-trophic species [81]. This strong selective deposition of DHA was also found for another LC-PUFA, DPA, and for one of the intermediates between LA and ARA, EDA, suggesting their incorporation into muscle phospholipids and the cellular membrane. In the FA biosynthesis pathway, EPA may have an intermediate role between DHA and ALA, reflecting dietary supply while still buffering low dietary intake, suggesting regulated partitioning between structural (membrane) and metabolic (eicosanoids precursor, partial oxidation) use. The presence of dietary EPA is suggested to reduce the deposition of ARA in many fish species [81]. EPA is also suggested to be elongated into PUFA such as DHA in freshwater or low-trophic species, but it is either β-oxidized for energy production or incorporated into cellular membranes or muscle for marine or high-trophic species, such as European sea bass [81]. A dose of retro-conversion of DHA into DPA through peroxisomal β-oxidation may also be involved [81,82] and would support the high levels of deposition of DPA. Interestingly, many studies have consistently reported that an adequate supply of OA (18:1 n-9, high in the RO diet), which is a primary substrate FA for triglycerides synthesis typically used for energy mobilization, could spare the PUFA from being β-oxidized for energy production [81]. This could explain the deposition of EPA and DHA in fish fed RO, slight for EPA and strong concerning DHA. Xu and colleagues [81] have also suggested that when supplied in excess, DHA and EPA are oxidized for energy production, and this could have been the case for EPA in the FO diet, as fish fed this diet showed slightly decreased levels of EPA compared to dietary levels.

Overall, the fatty acid profiles of European sea bass reflected hierarchical regulation of lipid metabolism. Collectively, these findings demonstrate that this marine fish species regulates fatty acid composition through selective retention rather than passive incorporation, with clear prioritization of long-chain highly unsaturated FA, especially EA, EDA, DPA, and DHA over C18 precursors (OA, LA, ALA) mainly used for energy [81]. Intermediate FA such as SDA, ARA, and EPA are maintained through metabolic partitioning between selective retention and utilization.

The investigation of the efficacy of both finishing diets ROFO and ROAO has shown that the reductions of ARA, EPA, and DHA in RO-fed fish (−32.4, −31.0, and −38.0%, respectively) compared to FO-fed fish for 25 wk suggested almost complete recovery (−2.9, −7.1, and −5.0%, respectively) when fish were fed the FO diet for the last 12 wk of the trial. Feeding the 13 wk RO-fed fish the ARA-rich AO diet for the last 12 wk only partly reversed

EPA and DHA to reduction levels of 16.7 and 16.5%, respectively, and even exceeded ARA levels found in FO-fed fish (+20.6%). Other studies in Mediterranean fish, European sea bass [27,28,66], gilthead seabream [12,21,25,83,84], red seabream, *Pagrus auratus* [24], or Mediterranean yellowtail, *Seriola dumerili* [22], have shown partial to complete recovery of DHA and ARA, while EPA was often only partially recovered. The partial recovery of DHA and EPA obtained in AO-fed fish may be linked to the slightly lower dietary levels of DHA and EPA in the AO diet compared to the FO diet. A microalgae-based diet slightly richer in LC-PUFA may have been as efficient as the FO diet and should be investigated in future experiments.

From a human nutritional perspective, seven indices have been calculated from the FA profile to assess the nutritional quality of the lipid fraction of the fish fillets. High levels of n-3 PUFA in fish fillets are desirable due to their health benefits, including cardioprotective effects and improved immune function and mental and metabolic health [85,86]. The Recommended Daily Intake (RDI) of EPA + DHA is estimated at 0.25 g per day for healthy individuals [87]. Despite the observed changes in fish fillet FA composition, the EPA + DHA levels at the end of the growth period of fish fed the AO or RO diets were slightly reduced compared to those of fish fed the FO control diet but were still much higher than the RDI. The finishing process with the FO or AO diets for 12 wk after the previous 13 wk of feeding with the RO diet increased the amount of EPA + DHA, making these fillets better than RO-fed fish in terms of nutritional value of the end products. The n-3/n-6 ratios obtained in the present study were close to those obtained by Montero and colleagues [27] when 60% of the FO was replaced by RO. The 35.7% reduction in the n-3/n-6 ratio of the RO group was fully restored when fish were fed an FO-based diet for 12 wk prior to commercialization, but a switch to a microalgae-based diet only mitigated the reduction to 21.4%. The recovery of the n-3/n-6 ratio by a finishing FO diet was also shown in *S. dumerili* [22]. Another important index is the Unsaturation Index (UI), with high values reflecting the ability of fish to maintain membrane fluidity at relatively low temperatures [55]; while the fish lipid quality (FLQ), a quality index designed specifically for fish, focuses on DHA and EPA in relation to SFA. Both these PUFA are very important for human brain function and reduce the risk of cardiovascular disease and dementia [85,86,88]. Previous studies have reported the FLQ indices ranging from 1.3 to 36 in different fish species [55,89]. In the present study, it was particularly high, ranging from 50 in RO-fed fish to 69 in FO- and AO-fed fish, making them a particularly healthy choice for human consumption. As expected, both UI and FLQ were lower in fish fed with the RO diet for 25 wk compared to the FO and AO diet groups but tended to be reversed in RO-fed fish after the 12-wk finishing process whether performed with FO or AO. Three supplementary FA quality indices related to human health cardio-protection were calculated namely the Hypocholesterolaemic/hypercholesterolaemic (h/H) ratio, the atherogenicity (AI) and the thrombogenicity indices (TI). These indices focus on the ratio of SFA (favouring lipid adhesion to circulating and immune cells) and unsaturated FA (inhibiting plaque accumulation and reducing the levels of phospholipids, cholesterol and esterified fatty acids) [55,56]. Low AI and TI values reflect food products with strong cardiovascular protection whereas high h/H ratio reflects a good lipid quality [55]. Low levels of SFA, principally 16:0, were obtained in fish fed the AO and RO diets for 13 wk compared to fish fed the FO diet (−4.7 and −6.2%, respectively) and even more so after 25 wk (−8.2 and −13.1%, respectively). These results were not surprising as fat of animal origin contains higher levels of SFA compared to those extracted from plants. Reduction in SFA was also observed in sea bass fed plant oils [28] and in gilthead seabream fed microalgae [49]. As expected, the switch from RO to FO or AO diets resulted in less pronounced SFA reduction after 25 wk (−5.9 and −8.6%, respectively). Thus, fish fed the AO diet and even more so the RO diet showed significantly higher h/H and lower AI than

fish fed the FO diet while finishing diets brought fish fillets to intermediate levels of these two indices. The same was true for Mediterranean yellowtail [22]. However, it should be noted that fish fed the FO diet in the present study had a h/H of 3.59 towards the high end of the normal range obtained in fish species (0.21–4.83) [55,89] and an AI of 0.23, not as good as that of RO-fed fish (0.18) but which can still be considered as "healthy" compared to foods high in saturated fat such as meat (AI up to 1.32) or dairy products (AI ranging from 1.42 to 5.13) [55]. TI values were comparable in the experimental groups examined herein (0.23–0.25), but also in Mediterranean yellowtail white muscle (0.23) [22] and in many other marine fish species [90], and was within the recommended values for human health (<1.0) [91,92]. In comparison, TI ranged from 0.19 to 0.63 in juvenile sea bass fed with increasing amounts of animal fat [93].

The Principal Component Analysis has been shown to be a useful tool for understanding complex data such as fatty acids [94,95]. It was therefore used in the present study to better understand and visualize the effect of the fish diets on the fatty acids or quality indices of the fish fillets. This analysis showed that the FA profile of FO-fed fish was mostly defined by their richness in 16:00, EPA, DPA, DHA and 16:1 n-7, while RO-fed fish were skewed towards OA, LA, ALA, and 18:3 n-6, 20:1 n-9 and 22:1 n-9. AO-fed fish showed a FA profile mainly defined as ARA- rich. The finishing diet ROAO recentered the FA profile towards the profile of RO-fed fish while ROFO skewed the FA profile more strongly towards that of FO-fed fish rich in DHA, DPA and EPA. Taking into account all the FA showing significant differences between the diets, 77.8% of the differences among diets were explained, while significantly different quality indices, i.e., all indices except for TI, explained 95.9% of these differences in the FA profile of the fish fillets. FOFO-fed fish were skewed towards high AI and SFA mainly due to high levels of EPA, DHA and their intermediate docosapentaenoic acid (DPA, 22:5 n-3), while RORO-fed fish were more determined by n-9 MUFA (mainly due to high OA) and n-3 and n-6 PUFA (mainly due to high ALA and LA). AOAO-fed fish showed an intermediate position between FOFO- and RORO-fed fish with high levels of ARA. ROAO- and even more so ROFO-fed fish were skewed from the RORO position towards the position of FOFO-fed fish. This clearly shows the efficacy of the finishing strategy with AO and especially with FO to mitigate the effects of long-term feeding of the fish with RO and to restore the FA profile of the fish fillet close to that of fish fully fed with FO. A longer finishing period may have allowed full recovery of the healthy FA profile.

4.4. Fish Liver and Intestine Histology

Histological examination suggested a greater accumulation of lipid droplets in the livers of fish fed the RO diet than in fish fed the FO and AO diets, consistent with a possible effect of dietary lipid source on hepatic lipid deposition. Similar hepatic steatosis has been reported in European sea bass and gilthead seabream fed RO [12,28], and in Nile tilapia fed *Nannochloropsis oculata* [71]. In contrast, fish fed 7% of *Nannochloropsis* sp. and 3% of *Schizochytrium* sp. appeared to exhibit less hepatocyte vacuolation than RO-fed fish. Likewise, fish switched from the RO diet to FO or AO during the finishing period appeared to show a reduced hepatic lipid accumulation characterised by a mosaic pattern rather than uniform vacuolisation throughout the liver section. This observation was consistent with the lower HSI and VSI values observed in these groups. Overall, the histological observations suggested that the increased lipid accumulation associated with the RO diet may have been partially reduced following the finishing strategy with AO or FO-based diets. However, as the histological assessment was qualitative, these observations should be interpreted with caution.

Qualitative examination of the distal intestine suggested slightly greater leukocyte infiltration in RO-fed fish than in FO- or AO-fed fish, in agreement with previous observations in European sea bass [28]. These slight differences may have been due to the rich oleic acid content of the RO diet. This FA has been linked to antioxidant defense mechanisms at the intestinal level of European sea bass [96]. However, these differences were no longer apparent at the end of the trial. Overall, the distal intestine displayed normal histological features across all dietary treatments.

4.5. Fish Haematological and Immune Parameters

Long-chain n-3 PUFA (EPA and DHA) influence health through four main mechanisms, (i) competing with ARA to reduce pro-inflammatory eicosanoids production, (ii) serving as precursors of specialized pro-resolving mediators; (iii) regulating signaling pathways and gene expression via cellular and nuclear receptors, (iv) altering membrane fluidity, thereby affecting membrane-bound enzymes, receptors and ion channels. Together, these mechanisms strongly modulate inflammatory and immune responses [97–99]. Despite clear differences in dietary FA composition, no significant effects were observed on hematological or immune parameters tested after 25 weeks, suggesting that all diets adequately supported immune function. Similar findings have been reported in gilthead seabream fed 5 and 10% *Nannochloropsis* for 2–4 weeks [100] and in seabream and Atlantic salmon fed rapeseed oil-based diets [101]. Although dietary lipids can influence respiratory burst activity and phagocytosis, responses vary among species and environmental temperature [28,97,101–104]. In the present study, myeloperoxidase activity was unaffected, while ceruloplasmin activity tended to be higher in fish fed reduced FO diets, consistent with reports of increased inflammatory markers following reduced DHA and EPA [105] or microalgae inclusion [71].

Similarly, dietary oil source had little effect on antibacterial immune responses. Lysozyme activity was not affected, in agreement with previous studies in Atlantic salmon and European sea bass [28,106], although it tended to be higher in fish fed the RO-based diet. Complement activity against *E. coli* tended to be increased in fish fed the AO-based diet than in fish fed the FO-based control diet, suggesting a modest immunostimulatory activity which could be due to bioactive compounds of microalgae such as polysaccharides [107,108]. Previous studies have likewise reported slight immune stimulation in Atlantic salmon fed *Nannochloropsis gaditana* and *Schizochytrium* spp. [109], alterations in sea bass gut microbiota with *Nannochloropsis* inclusion [110], and improved immune responses and and disease resistance in *Labeo rohita* fed low levels of a mixture of microalgae [111], supporting the potential of microalgae as functional aquafeed ingredients.

5. Conclusions

In conclusion, long-term dietary lipid source had little effect on fish growth, feed efficiency, fillet composition or overall health, but finishing diets significantly influenced morphometric indices, fillet FA profiles, liver histology and fish quality. Dietary rapeseed oil reduced SFA and n-3 PUFA (especially EPA, DPA and DHA) and increased MUFA and n-6 PUFA (especially OA and LA) in the fish fillet. However, tissue FA profiles were not a direct mirror of diet, indicating selective lipid deposition and metabolic regulation rather than a biosynthetic capacity, absent or extremely low in European sea bass. DHA and, to a lesser extent ARA, were strongly conserved, EPA showed an intermediate regulated deposition alongside metabolic utilization, while LA was moderately and ALA poorly retained, consistent with preferential catabolism. Overall, long-chain HUFA were preferentially incorporated into tissues. A 12-wk FO finishing period shifted the fatty acid profile of fish previously fed with the rapeseed oil diet toward that of fish continuously fed FO, whereas

the microalgae-based finishing diet produced a smaller shift, potentially due to the slightly poorer AO diet in LC-PUFA than the FO diet. These findings suggest that rapeseed oil and microalgae are promising alternatives to FO in a finishing diet strategy, substantially reducing reliance on FO over the full production cycle while maintaining fish performance and allowing recovery of a healthy FA profile before harvest.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes11080444/s1, Figure S1: Posterior intestine sections of fish fed (A) FO diet, (B) AO diet and (C) RO diet for 13 wk (Latin letters 10× Greek letters 20×). EG eosinophilic granulocytes. n = 9. Figure S2: Posterior intestine sections of fish fed continuously (A) FO diet (FOFO), (B) AO diet (AOAO), (C) RO diet (RORO) for 25 wk, or of fish fed RO for 13 wk and switched to (D) FO diet (ROFO) or (E) AO diet (ROAO) 13 wk after the start of the trial (10×). n = 6. Figure S3: Dietary FA profile of selected FA and comparison with the fillet FA profile of European sea bass after 13 or 25 wk feeding with the 3 different experimental diets (FO, AO, RO) or after 12 wk of finishing with the FO or AO diets in fish fed the RO diet for the first 13 wk (ROFO, ROAO). ALA = α-linolenic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EA = erucic acid; EDA = eicosadienoic acid; EPA = eicosapentaenoic acid; HDA = hypogenoic acid; LA = linoleic acid; OA = oleic acid; PA = palmitic acid; POA = palmitoleic acid; SA = stearic acid; SDA = stearidonic acid.

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Abbreviations

The following abbreviations are used in this manuscript:

ADF	Acid Detergent Fiber
AI	Atherogenic index
ALA	α-linolenic acid
ARA	Arachidonic acid
AO	Algal oil
BW	Body weight
CF	Crude Fiber
CP	Crude protein
DFI	Daily Feed Intak
DHA	Docosahexaenoic acid
EA	Erucic acid
EDA	Eicosadienoic acid
EE	Ether extract
EPA	Eicosapentaenoic acid
ERA	Eicosatrienoic acid
FA	Fatty acids
FCR	Feed Conversion Ratio
FLQ	Fish lipid quality
FM	Fishmeal
FO	Fish oil
HDA	hypogenoic acid
h/H	Hypocholesterolaemic/hypercholesterolaemic ratio
HSI	Hepatosomatic Index
LA	Linoleic acid
LC-PUFA	long-chain polyunsaturated fatty acids
MFI	Mesenteric fat index
MUFA	Monounsaturated fatty acids
NDF	Neutral Detergent Fiber
NFE	Nitrogen-Free extract
OA	Oleic acid
OM	Organic Matter
PA	Palmitic acid
POA	Palmitoleic aid
PUFA	Polyunsaturated fatty acids
RO	Rapeseed oil
SA	Stearic acid
SDA	Stearidonic acid
SGR	Specific Growth Rate
SFA	Saturated fatty acids
TI	Thrombogenic index
UI	Unsaturation index
VSI	Viscerosomatic Index
WG	Weight Gain
Wf	Final weight
Wi	Initial weight
wk	weeks

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