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Effect of dietary inclusion of camelina or chia oil on fatty acid digestibility, histology, blood biochemistry and molecular biomarkers in juvenile gilthead sea bream (*Sparus aurata,* L.)

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Abstract

The present study evaluated fatty acid digestibility, histology, blood biochemistry and gene expression in the gut of juvenile gilthead sea bream, *Sparus aurata* (initial mean weight, 60.4 ± 4.9 g) fed with iso-proteic (48%) and iso-lipidic (20.7%) diets in which fish oil was partially (60%) or totally (100%) replaced with either camelina or chia oil. Each of the experimental diets was fed to triplicate groups of fish stocked at 10 fish/tank in 150 L tanks for 90 days. Although inclusion of camelina or chia oil altered dietary fatty acid (FA) profile, major differences in digestibility in the individual FAs were not observed. Total replacement of fish oil with camelina or chia oil did not have any effect on blood glucose levels, whereas cholesterol and triglyceride levels were reduced. Results of messenger ribonucleic acid (mRNA) transcription showed elevated levels in the expression of the elongases and desaturases in gilthead seabream fed with diets devoid of fish oil. Fish fed with camelina or chia oil based diets had increased expression in lipolytic and lipogenic genes, resulting in accumulation of lipid droplets in the gut of fish. Histological examination revealed no pathological disorders in the guts of the fish.

Introduction

The search for alternatives to wild fishery-derived raw materials including fish oil has intensified due to the stagnation in the supply and the continuous increase in the demand (Turchini *et al*., 2009; Tocher, 2015). To mitigate this problem, researches have shifted to increased dietary inclusion of products from plant origin in fish diets. However, these plant-derived products lack the highly unsaturated fatty acids (HUFAs, C≥20; n≥3) such as eicosapentaenoic (EPA, 20:5*n*-3), docosahexaenoic (DHA, 22:6*n*-3) and arachidonic (ARA, 20:4*n*-6) acids, which are essential for optimal growth and the maintenance of good health in animals including fish. Hence, dietary inclusion of vegetable oil (VO) creates new challenges such as dietary deficiency in HUFAs and unbalanced *n*-3/*n*-6 fatty acid (FA) profiles (Tocher, 2015). This may be the cause of the adverse effects on growth, organ functionality and physiology; welfare and well-being as well as influence on mRNA expressions in fish (Caballero *et al*., 2004; Krogdahl *et al*., 2010; Bentacor *et al*. 2016). In order to continue the current trend of increased substitution of marine ingredients such as fish oil, it is critical that the understanding of the extent to which these dietary manipulations have on fish physiology and welfare is deepened.

Gilthead sea bream (*Sparus aurata*) is a major cultured finfish in the Mediterranean area. Earlier studies demonstrated that up to 70% of dietary fish oil can be replaced with single or blends of VO in practical diets for juvenile gilthead sea bream without compromising growth and health when fish meal constituted 15-20% of dry matter (Benedito-Palos *et al.*, 2008, 2009, 2016; Fountoulaki *et al*., 2009). Conversely, recent studies have shown that higher levels of VO can replace fish oil in this species when fed with diets which contain fish meal being ≥40% of the dietary ingredients (Bouraoui *et al*., 2011; Betancor *et al*., 2016).

The extent to which a fish species can utilize FAs in VO to satisfy its energy needs is dependent on its position on the trophic level. Fishes occupying low trophic levels (thus, trophic levels < 3) can readily utilize dietary α-linolenic acid (ALA, 18:3*n*-3) and linoleic acid (LA, 18:2*n*-6) whereas those higher up the trophic level (trophic levels 4 and 5) predominantly require HUFA to satisfy their essential FA requirements. The inclusion of VO at increasing levels (>70% of dietary lipid content) in the diets of marine carnivores was reported by some authors to cause deficiency in HUFA and unbalanced *n*-3/*n*-6 fatty acid (FA) in addition to reduced growth (Izquierdo *et al*., 2005; Benedito-Palos *et al*., 2008). However, Ofori-Mensah *et al*. (2020a) in a recent study clearly demonstrated that gilthead sea bream, a marine carnivore occupying trophic levels between 3 and 4, can derive some of its metabolic energy needs from dietary ALA and LA. Hence, good growth was reported in this species when camelina and chia oils totally replaced fish oil in a diet containing 38% of fish meal (Ofori-Mensah *et al*., 2020b).

It has been reported in some studies that replacement of fish oil with VO causes changes in the histology of major tissues as well as accumulation of lipids in the vacuoles of the liver and lipoid liver disease in this species (Wassef *et al*., 2007; Benedito-Palos *et al*., 2008; Fountoulaki *et al*., 2009). Others have reported the accumulation of lipid droplets in the enterocytes of gilthead sea bream fed with diets containing increased levels of fish oil replacements (Caballero *et al*., 2003; Ballester-Lozano *et al.*, 2015). These studies revealed that the histological changes signify metabolic disorders arising from defective supply in dietary phospholipids or HUFA. Dietary lipids could regulate gene expression and the activity of key enzymes and transcription factors involved in anabolic and catabolic processes during lipid metabolism (Castro *et al*., 2016). It has been demonstrated that replacing fish oil with VOs could regulate the activity of enzymes involved in lipogenesis (Panserat *et al*., 2008; Morais *et al*., 2011, 2012; Peng *et al*., 2014) or oxidation (Ji *et al.*, 2011; Zuo *et al*., 2013). Such dietary manipulation may alter the normal functioning of enzymes involved in fatty acid synthesis, oxidation, uptake and storage in these internal organs. Therefore, the aim of the present study was to evaluate the effect of partial or total replacement of fish oil with camelina or chia oil on fatty acid digestibility, histology and gene expression in the gut of juvenile gilthead sea bream. In addition, the study assessed the effect of the dietary treatments on blood biochemistry.

Materials and Methods

Experimental Diets

Five iso-proteic (48%) and iso-lipidic (20.7%) were formulated with the control diet containing fish oil (FO diet). Camelina oil and chia oil totally replaced fish oil in CSO and CO diets, respectively, whereas a combination of fish oil (40%) and camelina oil or chia oil (60%) constituted 60CSO and 60CO diets, respectively. Fish meal (≈ 18% dry matter) was added to each diet to

Table 1. Formulation and proximate composition of experimental diets for juvenile gilthead sea bream

Feed Ingredients (g/kg)	FO	CSO	CO	MIX1	MIX2
Fish meal	382	382	382	382	382
Corn gluten	278	278	278	278	278
Soya meal	40	40	40	40	40
Wheat meal	40	40	40	40	40
Gelatin	40	40	40	40	40
Fish oil	180	0	0	72	72
Camelina oil	0	180	0	108	0
Chia oil	0	0	180	0	108
Mineral premix[a]	15	15	15	15	15
Vitamin premix[a]	15	15	15	15	15
Cr_2O_3	10	10	10	10	10
Analyzed Proximate Composition(g/kg)					
Dry matter	921	921	922	924	926
Crude Protein	483	482	483	484	486
Crude Lipid	206	205	206	209	205
Ash Content	114	115	114	114	116
Crude Cellulose	19	19	19	19	19
N-FE	99	99	100	98	99

FO = fish oil, CSO = camelina seed oil, CO = chia oil, 60CSO = 60% CSO+40% FO, 60CO = 60% CO+40% FO.
[a]Premix of vitamins and minerals according to NRC (2011) recommendations for fish.
N-FE, nitrogen-free extracts.

supply limited amount of dietary HUFA. The dietary ingredients and proximate compositions of diets are presented in Table 1.

FA composition of the experimental diets is shown in Table 2. The proportion of total saturated fatty acids (SFA) was highest in FO diet, of which palmitic acid (PA, 16:0) formed the bulk of the saturates. Monounsaturated fatty acids (MUFA) were higher in CSO diet. Within MUFA, high levels of oleic acid (OA, 18:1*n*-9) and eicosenoic acid (20:1*n*-9) were recorded in the dietary groups. Among the dietary groups, CO diet had the highest dietary level of 18: *n*-3 and 18:2*n*-6. In general, *n*-3 and *n*-6 polyunsaturated fatty acids (*n*-3, *n*-6 PUFA) were higher in camelina and chia oil based diets, while FO diet had higher levels of EPA, DHA and ARA.

Experimental Conditions

The Institutional Animal Care Committee of the Mediterranean Fisheries Research Production and Training Institute approved in advance all procedures (Protocol number 68385072-325.04-0967) used in this study. The study was conducted at the Research Unit of the Mediterranean Fisheries Research Production and Training Institute, Beymelek, Antalya, Turkey, after 2 weeks of acclimation to experimental conditions. Gilthead sea bream (*Sparus aurata*) of initial mean weight 60.5±4.9 were stocked at 10 fish tank^{-1} in triplicate groups in a flow-through system equipped with 15 cylindro-conical experimental tanks (150 L capacity), supplied with continuously aerated filtered seawater at a rate of 12 L min^{-1} and at a temperature of 27.8±0.7°C. The fish were fed with the experimental diets to apparent satiation by hand twice per day at 0900 and 1600 h for 90 days. At the end of the feeding trial, all fish were not fed for 24 hours and afterwards they were weighed individually after mild anesthesia (benzocaine administered at 35 mg L^{-1})

Fatty Acid Digestibility

Apparent digestibility coefficient of fatty acids (ADC_{FA}) in the experimental diets were calculated as:

$$ADC_{FA} = 100 - [100\ (Cr_2O_3 \text{ in diet}) \div (Cr_2O_3 \text{ in faeces}) \times ((\%\ FA \text{ in faeces}) \div (\%\ FA \text{ in diet})]$$

Histological Examination

In order to evaluate the effects of the camelina and chia oils on the digestive system of the experimental fish, histological examination was carried out by excising intestines of four (4) fish per tank after dissection on chilled trays. The intestine samples were processed for histological examination by fixing in 10% buffered formalin, and processed for paraffin embedding.

Table 2. Fatty acid composition (% FAME) of formulated diets

Fatty acid	FO	CSO	CO	MIX1	MIX2
14:0	7.2	3.2	2.7	5.8	4.9
16:0	18.4	7.6	8.4	10.9	11.5
18:0	4.1	2.9	3.9	3.1	3.2
20:0	0.3	1.1	0.2	0.6	0.2
22:0	0.8	1.2	0.1	0.9	0.4
24:0	0.2	0.3	0.1	0.2	0.1
∑SFA	31.6	16.5	15.7	22.3	20.7
16:1*n*-7	4.1	1.4	1.9	2.3	2.4
18:1*n*-7	2.6	1.3	1.3	1.7	1.8
18:1*n*-9	15.6	18.3	5.6	15.5	8.5
20:1*n*-9	6.4	10.1	1.1	7.8	1.4
24:1*n*-9	0.4	0.4	0.1	0.3	0.2
∑MUFA	29.1	31.5	9.9	27.5	14.4
18:2*n*-6	10.0	14.5	15.3	13.5	14.5
20:2*n*-6	0.4	0.1	0.2	0.4	0.4
18:3*n*-6	0.3	ND	ND	0.2	0.2
20:3*n*-6	0.8	ND	ND	0.4	0.4
20:4*n*-6	0.7	0.4	0.4	0.5	0.5
∑*n*-6 PUFA	12.2	15.0	15.8	15.1	16.0
18:3*n*-3	4.8	31.6	52.5	23.6	36.7
20:3*n*-3	1.2	ND	ND	0.2	0.2
18:4*n*-3	1.3	0.3	0.3	1.0	1.0
20:4*n*-3	1.2	ND	ND	0.7	0.7
20:5*n*-3	8.4	2.4	2.5	4.1	4.3
22:6*n*-3	11.4	3.4	3.6	5.7	5.8
∑*n*-3 PUFA	28.2	37.6	60.0	35.4	48.7
n-3/*n*-6	2.31	2.51	3.72	2.34	3.04
EPA + DHA	19.7	5.7	6.2	9.8	10.2

FO = fish oil, CSO = camelina seed oil, CO = chia oil, 60CSO = 60% CSO+40% FO, 60CO = 60% CO+40% FO.
ND = not detected.

Histological sections (4-5 µm) were stained using hemotoxylin and eosin (H & E) and then they were examined by light microscopy (Culling, 1963; Bullock, 1978)

Blood Biochemical Analyses

The experimental fish were not fed for 24 hours at the termination of the experimental feeding. Blood was drawn from the caudal peduncle with 1.0 mL syringe from 4 fish/tank (12 fish per dietary treatment) and sent to the laboratory for analysis. Biochemical parameters (glucose, creatinine, total protein, triglyceride, cholesterol, high density lipoprotein, low density lipoprotein, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase) of the blood were analysed with an automatic blood analyzer (Hitachi 7600-110 Ltd., Japan) at a clinical laboratory in Demre Hospital, Antalya, Turkey.

Ribonucleic Acid (RNA) Extraction

This was done according to Ofori-Mensah *et al.* (2020b). Total RNAs were isolated from anterior intestinal tissues using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to manufacturer 's protocol. RNA concentrations ranged between 500 and 1000 ng/µL with an absorbance ratio (A260/A280) of 2.0±0.15. Reverse transcription of 1 µg of total RNA was then subjected to the synthesis of the first strand cDNA with RevertAid® First Strand cDNA Synthesis Kit using random hexamer priming, according to manufacturer's instructions and stored at -26 °C until the RT-qPCR reaction occurred. The resulting cDNAs were further diluted with nuclease free-water (1:10) and it was subjected to quantitative real-time PCR analysis using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on a Roche LightCycler 480 real-time PCR system (Roche, Mannheim, Germany). PCR reactions on a total volume of 20 µl (consisted of 5 µl PowerUp SYBR Green Master Mix, 1.5 µl of each forward and reverse primers and 4 µl of cDNA) were carried out following standard cycling. *β*-Actin was used as a house-keeping gene and controls of PCR performance were included in each array. Negative controls without a template cDNA were routinely performed for each primer set. The primer sequences (Table 3) were designed using Primer 3 (version 0.4.0) software (http://bioinfo.ut.ee/primer3-0.4.0/). The relative expression of each gene was calculated according to the $2^{-\Delta\Delta}$ Ct method of Livak and Schmittgen (2001) and the housekeeping gene *β*-Actin was used to normalize the expression of other genes.

Data Analyses

The results of the present study are presented as mean±standard deviation (SD). All data were tested for normal distribution and homogeneity of variance using Levene's test prior to the other statistical analyses. In order to determine the main effect of dietary treatments, one-way analysis of variance (ANOVA) was carried out. This was followed by Tukey's post-hoc test (HSD) if dietary treatments had significant effect ($P \leq 0.05$) on a target parameter. All percentage data were arcsine transformed prior to analysis. All statistical analyses were performed using SPSS statistical software (Version 18).

Table 3. Real-time quantitative PCR primers for lipid related genes

Primer Code	Primer Forward	Primer Reverse
ELOVL1b	CTTCCTACACATCTTCCACCACTC	CCATTCCACCAGGAGCAAAGG
ELOVL4	CGGTGGCAATCATCTTCC	TCAACTGGCTGTCTGTGT
ELOVL5	CCTCCTGGTGCTCTACAAT	GTGAGTGTCCTGGCAGTA
ELOVL6	GTGCTGCTCTACTCCTGGTA	ACGGCATGGACCAAGTAGT
FADS2	GCAGGCGGAGAGCGACGGTCTGTTCC	AGCAGGATGTGACCCAGGTGGAGGCAGAAG
SCD1b	TAAAGTGGTGATGTTCCAGA	GCATCACAGTGTATCTGAGG
LPCAT1	GAAAGAAGACTGTGGAGGAG	TAAAGGTGATTAGGCAGGAT
FAS	TGCCATTGCCATAGCACTCA	ACCTTTGCCCTTTGTGTGGA
CEL	GCTGAGGAGATTGCTCTGAAGGT	CAGGAAGCCATAGTCTCACCAGTG
HSL	AGAGATGAGGTTGCAGAGTT	CAAGCCACAATATGTACAGG
LPL	CGTTGCCAAGTTTGTGACCTG	AGGGTGTTCTGGTTGTCTGC
CPT1a	GTGCCTTCGTTCGTTCCATGATC	TGATGCTTATCTGCTGCCTGTTTG
CPT1b	CCACCAGCCAGACTCCACAG	CACCACCAGCACCCACATATTTAG
SREBP1	AGGGCTGACCACAACGTCTCCTCTCC	GCTGTACGTGGGATGTGATGGTTTGGG
PPARα	TCTCTTCAGCCCACCATCCC	ATCCCAGCGTGTCGTCTCC
PPARβ	CAGGTGACTTTGCTGAAGTA	TAAAGGGCTTTCTTAAGCTG
PPARγ	CTCAAGAGTCTCAGGAAACC	GATAATGACAGCCAGAAACA
COX2	GAGTACTGGAAGCCGAGCAC	GATATCACTGCCGCCTGAGT
β-actin	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATGCT

ELOVL1b fatty acid elongase 1b; ELOVL4 fatty acid elongase 4; ELOVL5 fatty acid elongase 5; ELOVL6 fatty acid elongase 6; FADS2 fatty acid desaturase 2; SCD1b stearoyl-CoA desaturase 1b; LPCAT1 lysophosphatidylcholine acyltransferase 1; FAS fatty acid synthase; CEL carboxyl ester lipase; HSL hormone sensitive lipase; LPL lipoprotein lipase; CPT1a carnitine palmitoyltransferase 1a; CPT1b Carnitine palmitoyltransferase 1b; SREBP1 sterol regulatory element binding protein 1; PPARα peroxisome proliferator-activated receptor α; PPARβ peroxisome proliferator-activated receptor β; PPARγ peroxisome proliferator-activated receptor γ; COX2 cytochrome C oxidase 2.

Results

The experimental diets were readily accepted by the experimental fish and no mortality was recorded during the feeding trial. Good growth was recorded as initial weights were more than doubled after the 90-day feeding trial. However, fish fed with the CSO diet (diet in which fish oil was totally replaced with camelina oil) recorded reduced final mean weight.

The mean apparent FA digestibility in juvenile gilthead sea bream fed with the various experimental diets is shown in Table 4. Apparent digestibility coefficients (ADC) for FA were determined using chromic oxide as an internal marker. Digestibility values of the FA were above 70%. In general, digestibility was low in SFA, from 85.9 – 89.3% with fish fed 60CO diet recording the highest value. Among the MUFAs, 20:1*n*-9 had the highest digestibility value, and no significant differences were recorded among the different dietary treatments (P>0.05). The ADC for the MUFAs was between 87 and 93.6%. In comparison to the SFA and MUFA, digestibility was highest in the PUFA. Considering the *n*-3 and *n*-6 PUFAs, digestibility was comparatively higher in *n*-3 PUFA. Among the *n*-3 PUFA, (DHA) recorded the highest digestibility values whereas 20:3*n*-3 recorded the least value. For 18:3*n*-3, the highest digestibility was recorded in 60CO fed fish whereas fish fed with CSO diet recorded the least digestibility. Digestibility for dietary DHA was comparatively high in fish fed with chia oil-based diets (98.6% and 96.5% for CO and 60CO, respectively). Among the *n*-6 PUFA, digestibility was highest for 18:3*n*-6 (≈ 100%) whereas 18:2*n*-6 recorded the least value. The lowest digestibility in 18:2*n*-6 (84.4%) was recorded in fish fed with FO diet. Similarly, FO fed fish had the lowest digestibility in ARA (88.9%) whereas fish in 60CO dietary group recorded the highest value (94.2%), although not statistically different from 60CSO (94.0%) fed fish. In general, ADC followed the trend PUFA > MUFA > SFA.

Evaluation of the gut sections of fish fed with the different dietary treatments revealed no pathological disorders in the foregut (Figure 1). Intense accumulations of lipid droplets were observed in the foregut sections of both CO and CSO fed fish, especially at the tips of the villi in fish fed with the CSO diet. Foregut sections of fish fed with 60CSO diet had lipid droplets around the enterocytes. Although 60CO fed fish had lower lipid droplets, an expansion in the lamina

Table 4. Fatty acid digestibility (%) in juvenile gilthead sea bream fed experimental diets.

Fatty acid	FO	CSO	CO	60CSO	60CO
14:0	85.9±0.2	87.1±0.1	87.6±0.1	88.5±0.4	89.3±0.1
15:0	92.7±0.3^{b}	91.9±0.4^{bc}	95.4±0.3^{a}	94.4±0.1^{a}	91.0±0.4^{bc}
16:0	84.5±0.3	85.3±0.4	86.9±0.1	86.6±0.1	87.5±0.5
17:0	91.9±0.6^{b}	94.2±0.6^{b}	98.9±0.1^{a}	73.8±0.9^{d}	85.4±0.6^{c}
18:0	83.9±0.1	84.4±0.1	85.3±0.1	83.5±0.1	85.1±0.4
20:0	81.1±0.7^{c}	81.2±0.4^{c}	84.1±0.3^{b}	86.5±0.1^{a}	87.4±0.2^{a}
21:0	91.6±0.1^{b}	91.2±0.1^{b}	97.9±0.1^{a}	89.7±0.3^{c}	87.6±0.8^{c}
24:0	71.8±0.98^{c}	81.0±0.3^{b}	80.5±0.5^{b}	82.5±0.4^{a}	84.3±0.3^{a}
16:1n-7	90.5±0.1	88.6±0.3	87.4±0.4	89.4±0.0	89.8±0.1
17:1	90.3±0.4^{c}	96.1±0.2^{b}	98.8±0.5^{a}	88.4±0. 9^{c}	88.6±0.6^{c}
18:1n-7	89.1±0.3	89.6±0.1	88.9±0.2	89.2±0.1	89.3±0.1
18:1n-9	80.6±0.2^{d}	91.0±0.1^{a}	87.7±0.1^{c}	88.4±0.3^{b}	85.6±0.1^{c}
20:1n-9	98.9±0.6	98.2±0.4	99.2±0.6	98.4±0.3	98.7±0.4
24:1n-9	82.3±0.2^{c}	83.4±0.3^{c}	86.9±0.3^{b}	91.9±0.1^{a}	87.3±0.3^{b}
18:2n-6	84.4±0.1^{b}	87.4±0.1^{b}	89.4±0.1^{a}	86.4±0.0^{b}	88.6±0.1^{a}
20:2n-6	95.8±0.1^{b}	99.1±0.0^{a}	96.9±0.1^{ab}	98.5±0.1^{a}	95.0±0.2^{b}
18:3n-6	ND	99.8±0.1	ND	ND	ND
20:3n-6	89.6±0.2^{b}	ND	ND	92.5±0.1^{a}	91.1±0.4^{a}
20:4n-6	88.9±0.2^{c}	90.1±0.6^{c}	91.6±0.3^{b}	94.0±0.1^{a}	94.2±0.1^{a}
18:3n-3	93.3±0.1^{a}	88.7±0.2^{b}	92.9±0.1^{a}	88.8±0.2^{b}	93.5±0.2^{a}
20:3n-3	90.5±0.1	ND	ND	87.9±0.7	91.4±0.3
18:4n-3	90.3±0.1	89.7±0.2	89.3±0.4	91.7±0.1	91.4±0.2
20:5n-3	95.5±0.1	93.9±0.5	95.6±0.1	95.7±0.3	96.4±0.1
22:6n-3	96.3±0.4^{b}	94.1±0.1^{b}	98.6±0.1^{a}	94.9±0.1^{b}	96.5±0.3^{a}
∑SFA	85.0±0.1	85.7±0.1	86.7±0.2	86.7±0.3	87.5±0.1
∑MUFA	88.1±0.3^{c}	93.6±0.3^{a}	88.9±0.1^{c}	91.7±0.1^{b}	87.6±0.1^{c}
∑PUFA	91.7±0.2^{a}	89.9±0.1^{b}	92.9±0.3^{a}	89.9±0.0^{b}	92.8±0.0^{a}
∑n3	95.1±0.5^{a}	90.7±0.3^{b}	94.2±0.2^{a}	91.0±0.1^{b}	94.2±0.2^{a}
∑n6	86.1±0.3	88.6±0.2	89.4±0.4	88.0±0.1	89.3±0.1
n-3 HUFA	95.9±0.2	93.6±0.6	96.9±0.1	95.2±0.1	96.4±0.2
EPA+DHA	96.0±0.2	94.0±0.3	96.9±0.1	95.3±0.1	96.5±0.2

Data are presented as mean ± S.E. Different superscript letters within a row denote significant differences among diets as determined by one-way ANOVA with Tukey's comparison test (P<0.05).

FO = fish oil, CSO = camelina seed oil, CO = chia oil, 60CSO = 60% CSO+40% FO, 60CO = 60% CO+40% FO.

ND = not detected.

propria was observed in the foregut sections. The integrity of mucosa, submucosa and enterocytes were intact in the foregut of fish fed with the FO diet. The enterocytes had centrally-located nuclei with no increase in the number of goblet cells. Furthermore, there were no lipid droplets in the portion of enterocytes facing the lumen.

Similar to foregut sections, no pathological changes were observed in the mid-gut sections of test fish (Figure 2). The number of goblet cells was seen to be increased in mid-gut sections of fish fed with the FO diet with few lipid droplets. Few lipid droplets were seen around enterocytes with non-centrally located nuclei in 60CSO and 60CO fed fish. In addition, enlarged lamina propria and shortened villi were observed in the mid-gut sections in 60CO fed fish. Accumulation of lipid droplets were seen in the mid-gut section of fish fed CO diet. Similarly, lipid droplets were observed in mid-gut sections of fish fed CSO diet, which were observed to be localized at the villi.

Accumulation of lipid droplets were not found in any of the hindgut sections of fish fed with the experimental diets (Figure 3). Fish fed with FO diet had hindgut sections with increased number of goblet cells whereas shortening in villi length and an increase in the number of goblet cells were found in the hindgut sections of fish fed CSO and 60CSO diets. Hindgut sections of fish fed 60CO diet had very few mucous cells and enlarged lamina propria with no lipid droplets whereas that of fish fed CO diet had few goblet cells.

Table 5. Biochemical parameters (mean ± SEM) of dietary groups

Parameters	FO	CSO	CO	60CSO	60CO
Glucose (mg/dl)	85.3±0.3	73.7±0.7	80.0±1.7	85.3±4.8	81.3±1.6
Creatinine (mg/dl)	0.14±0.01^{bc}	0.15±0.01^{bc}	0.21±0.01^{a}	0.11±0.01^{c}	0.16±0.01^{ab}
Total Protein (g/dl)	3.87±0.19	3.39±0.12	3.76±0.14	3.98±0.18	3.59±0.12
Triglyceride (mg/dl)	237±4.84^{ab}	195.33±3.71^{c}	201.67±2.73^{c}	246.67±3.71^{a}	225.0±2.89^{b}
Cholesterol (mg/dl)	234.67±3.33^{a}	192.0±8.74^{bc}	183.67±7.51^{c}	214.67±6.23^{ab}	218.33±5.82^{ab}
HDL (mg/dl)	30.1±3.1^{b}	36.9±2.4^{a}	38.57±4.1^{a}	26.87±0.9^{bc}	28.23±3.7^{b}
LDL (mg/dl)	28.0 ±1.26^{a}	15.67±0.7^{b}	18.37±0.72^{b}	19.33±0.82^{b}	19.07±0.83^{b}
Calcium (mg/dl)	14.1±0.6	13.4±0.3	13.62± 0.9	13.7±0.4	13.5±06
GOT (IU/L)	79.8±1.3^{a}	42.2±0.5^{c}	57.6±1.0^{b}	83.8±2.0^{a}	60.8±0.6^{b}
GPT (IU/L)	8.13±1.65	7.6±1.96	7.33±1.73	12.97±2.66	9.23±2.64
ALP (IU/L)	217.3±1.5^{c}	254.17±1.58^{b}	347.17±3.14^{a}	252.93±1.58^{b}	182.43±1.59^{d}

Data are expressed as mean (n=3) ± SE. Means with different superscripts within the same rows indicate statistical significance (P<0.05).
FO = fish oil, CSO = camelina seed oil, CO = chia oil, 60CSO = 60% CSO+40% FO, 60CO = 60% CO+40% FO.
HDL = high density lipoprotein, LDL = low density lipoprotein, GOT = glutamate oxaloacetate transaminase, GPT = glutamate pyruvate transaminase, ALP = alkaline phosphatase.

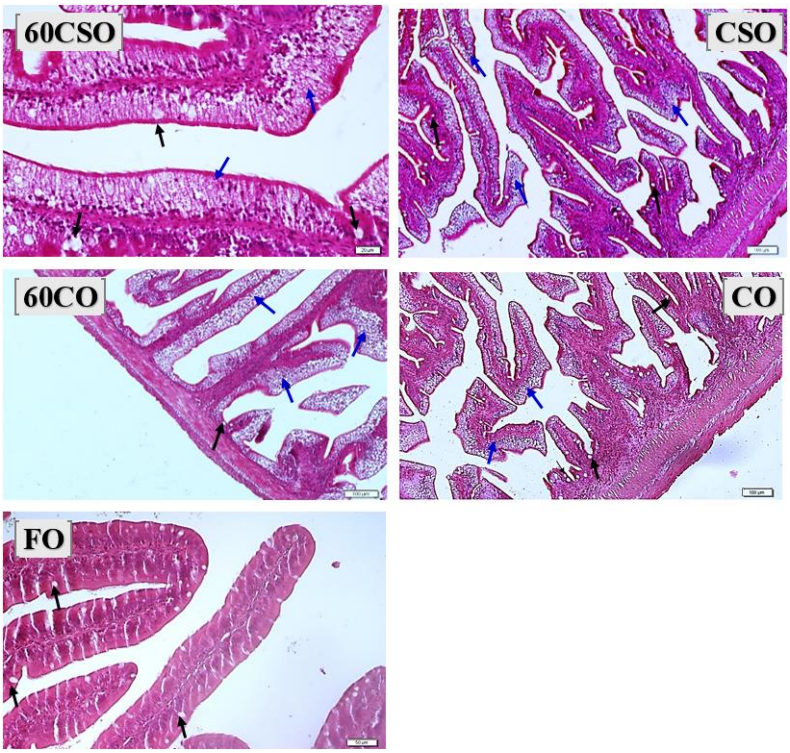

Figure 1. Foregut sections of fish fed the experimental diets with lipid droplets (blue arrows) and goblet cells (black arrows) around the enterocytes (H&E).

Results of mRNA expression of sterol regulatory element binding protein 1 (SREBP1), desaturases (FADS2 or Δ-6 desaturase; SCD1b or Δ-9 desaturase) and fatty acid synthase (FAS) in juvenile gilthead sea bream fed with the different experimental diets are shown in are shown in Figure 4. Compared to the other dietary groups, fish fed with diets containing chia oil recorded higher expression in SREBP1 gene. Fish fed with CO and CSO diets recorded higher levels in Δ-6 and Δ-9 desaturases whereas expression of FAS gene was lowest in FO fed fish.

For the elongases (ELOVL 6, 5, 4 and 1b), fish fed with the FO-devoid diets (CSO and CO) recorded high levels of ELOVL 6 in the gut while fish fed with diets

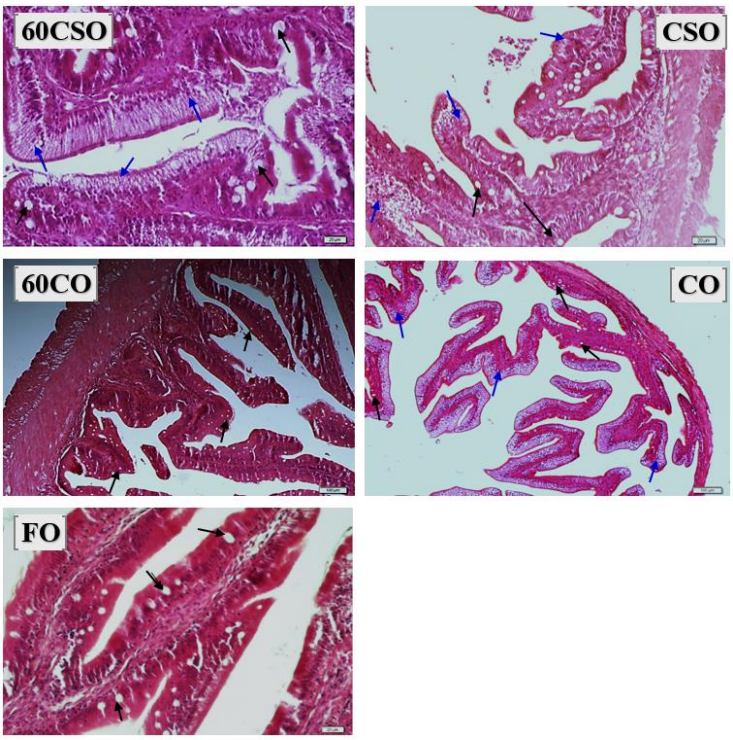

Figure 2. Midgut section of fish fed experimental diets with lipid accumulation (blue arrows) and goblet cells (black arrows) in the enterocytes (H&E).

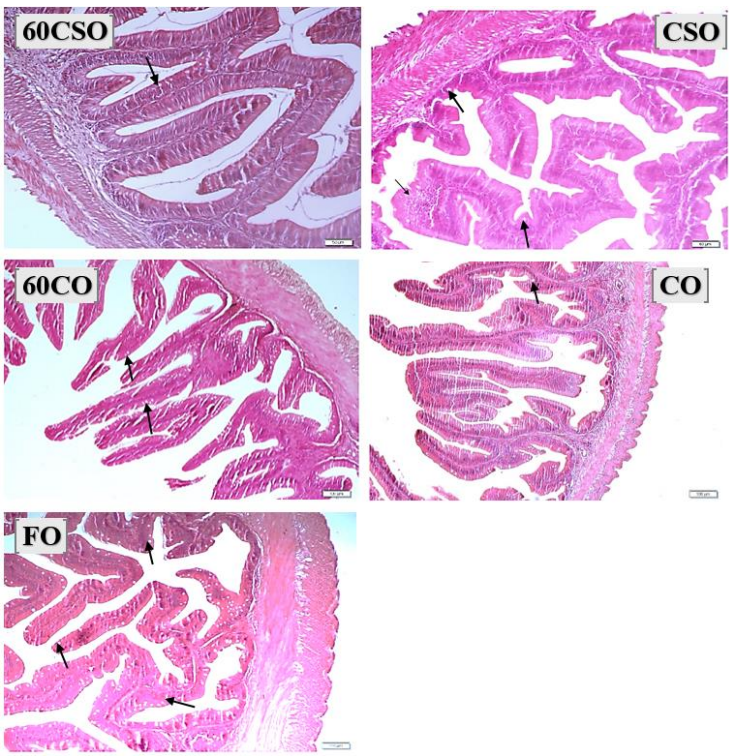

Figure 3. Hindgut sections fish fed experimental diets with goblet cells (black arrows) in the enterocytes (H&E).

containing fish oil showed low levels of expression (Figure 5). Likewise, feeding fish oil-based diets led to low level of expression of ELOVL4 in the anterior intestines of juvenile gilthead sea seabream. Compared to FO, 60CSO and 60CO fed fish, CSO and CO fed fish recorded higher levels of ELOVL1b.

Intestinal gene expression forlysophosphatidylcho line acyltransferase 1 (LPCAT1) (Figure 6) was low in gilthead sea bream fed FO and CO diets. For the lipases, carboxyl ester lipase (CEL) expression increased with increasing fish oil substitution, and consequently fish fed with the 100% VO diets recorded higher levels of expression. Fish fed fish oil-devoid diets recorded higher levels of expression of lipoprotein lipase (LPL) gene. For hormone sensitive lipase (HSL), CO, 60CSO and CO fed fish recorded higher levels of expression.

Feeding chia oil-based diets led to higher intestinal expression of carnitine palmitoyltransferase 1a (CPT1a) and carnitine palmitoyltransferase 1b (CPT1b) genes, whereas low and similar levels were expressed in fish fed FO, CSO and 60CSO diets (Figure 7). Similar levels of peroxisome proliferator-activated receptor α (PPARα)

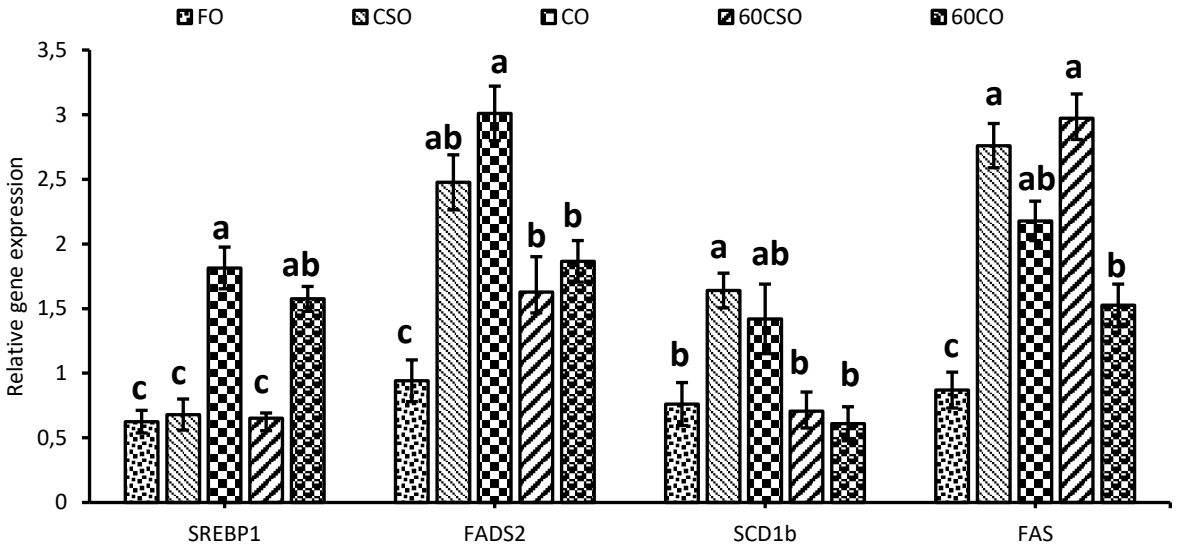

Figure 4. Effects of experimental diets on relative mRNA expression of SREBP1, FADS2, SCD1b and FAS in foregut of gilthead sea bream. Different letters denote significant differences among treatments (P<0.05).

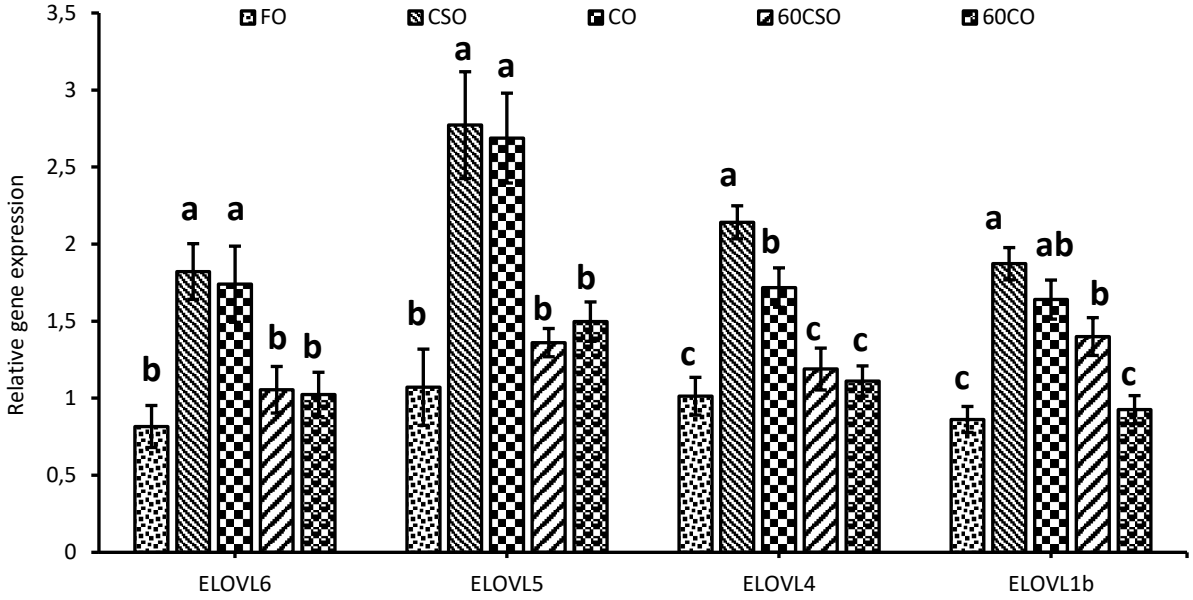

Figure 5. Effects of experimental diets on relative mRNA expression of ELOVL6, ELOVL5, ELOVL4 and ELOVL1b in foregut of gilthead sea bream. *Different letters* denote significant differences among treatments (P<0.05).

expression were recorded in CSO, CO, 60CSO and 60CO fed fish, which were higher than the level recorded by FO fed fish. FO fed fish recorded the lowest level of expression in peroxisome proliferator-activated receptor β (PPARβ). For peroxisome proliferator-activated receptor γ (PPARγ), the expression levels were higher in fish fed with diets containing a mixture of vegetable and fish oils (60CSO and 60CO). However, no significant differences were recorded in intestinal gene expression for cytochrome C oxidase 2 (COX2) in any of the fish fed with the experimental diets.

Table 5 shows the results of the biochemical parameters analysed in the present study. Glucose and total protein levels did not differ among the dietary groups whereas fish fed with diets containing chia oil recorded high levels of creatinine. Fish fed with CSO and CO diets recorded low levels of TAG, cholesterol and LDL-cholesterol but not HDL-cholesterol. There was no difference in blood content of calcium and GPT in all dietary groups. FO and 60CSO fed fish recorded high levels of GOT whereas CO fed fish had the highest ALP level ($P<0.05$).

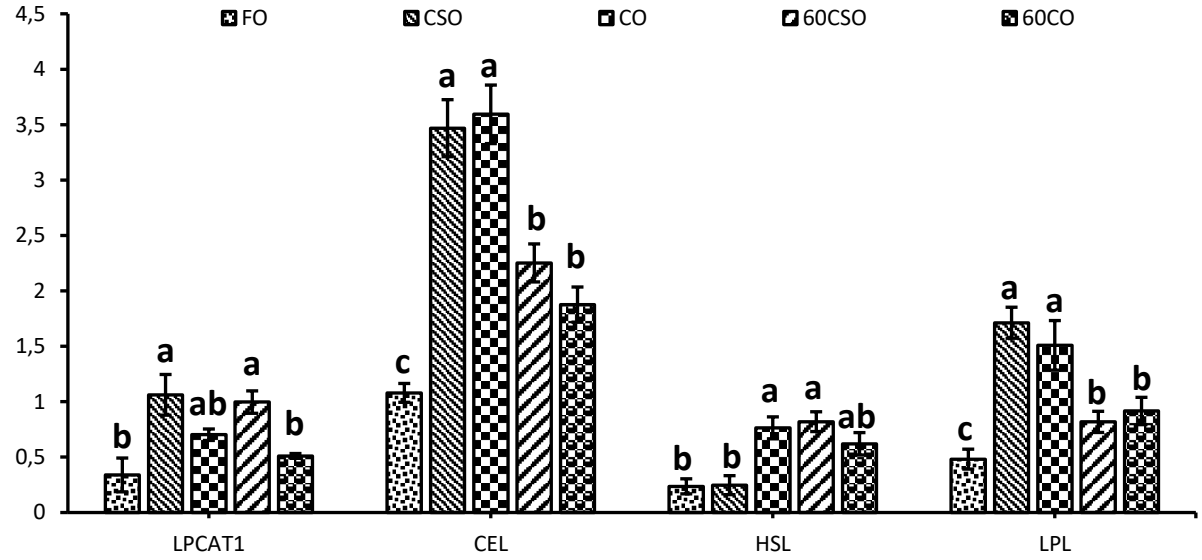

Figure 6. Effects of experimental diets on relative mRNA expression of LPCAT1, CEL, LPL and HSL in foregut of gilthead sea bream. Different letters denote significant differences among treatments ($P<0.05$).

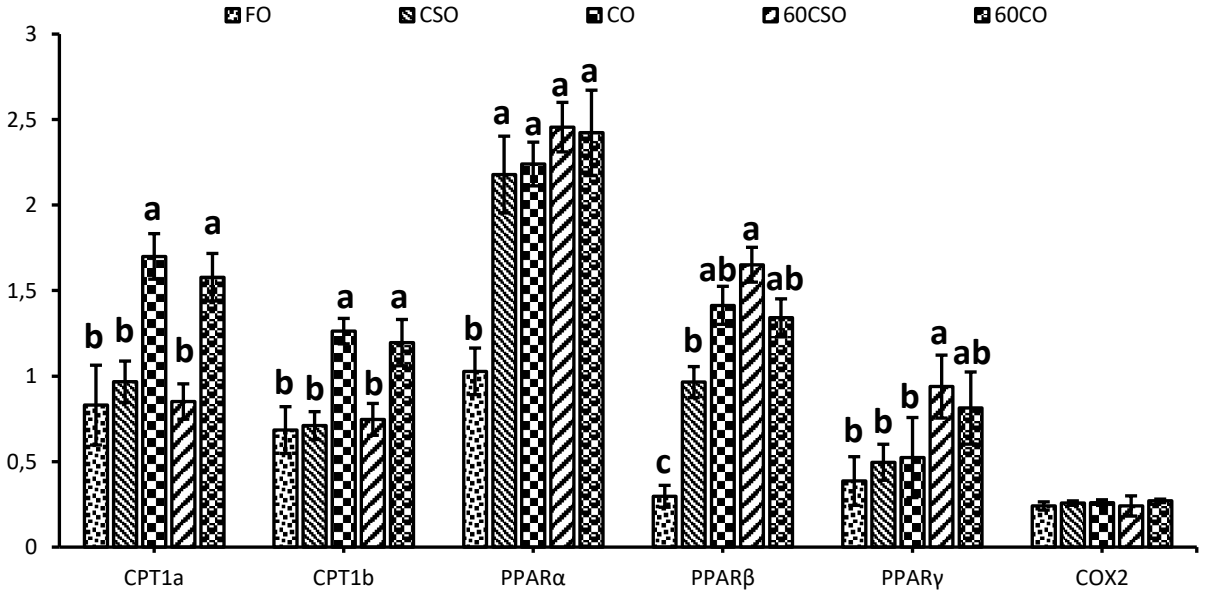

Figure 7. Effects of experimental diets on relative mRNA expression of CPT1a, CPT1b, PPARα, PPARβ, PPARγ and COX2 in foregut of gilthead sea bream. *Different letters* denote significant differences among treatments ($P<0.05$).

Discussion

Replacing FO with CSO and CO resulted in diets with high levels of ALA in the present investigation. CSO and CO have ALA levels up to 50 and 68%, respectively (Tocher *et al*., 2011), with appreciable levels of LA and OA, which resulted in elevated dietary levels of *n*-3 C_{18} PUFA and *n*-3/*n*-6 contents in diets containing camelina or chia oil in the present study. Conversely, inclusion of camelina and chia oil reduced dietary contents of DHA, EPA (eicosapentaenoic acid) and ARA (arachidonic acid) in the present study, as was reported in previous studies (Carbonera *et al*., 2016; Montanher *et al*., 2016). This is because, irrespective of their fatty acid profile, VOs lack HUFA. Hence, their inclusions in aquafeeds lead to reduced dietary content of DHA, EPA and ARA. Marked differences in the digestibility in individual FA were not recorded in the present study, similar to the results of previous studies (Betancor *et al*., 2015, 2016). In spite of this, digestibility of dietary FAs increased with increasing degree of unsaturation and decreased with chain length. Thus, digestibility followed a preferential order of PUFA > MUFA > SFA and short chain > longer chain fatty acids was demonstrated in the present study. Previous studies have reported similar trends for various fish species (Eroldoğan *et al*., 2013; Betancor *et al*., 2016).

Blood biochemistry parameters provide an insight into the nutritional, physiological and clinical status of animals, and they may be useful in the assessment of the suitability of feed and feeding practices, rearing conditions, presence of acute or chronic stressors and pathogenic manifestations of aquaculture species (Tewary & Patra, 2011; Peres *et al*., 2012). Reduced blood creatinine but not protein nor glucose was reported in sea bream fed with HUFA deficient diet (Ballester-Lozano *et al*., 2015). However, blood total protein, glucose and creatinine levels did not differ in a long term (31 weeks) study that evaluated the effects of concurrent and high substitution of fish meal and fish oil in gilthead sea bream diets (Benedito-Palos *et al*., 2016). Similarly, total protein and creatinine levels did not differ in gilthead sea bream fed with the various dietary treatments in the present study, and they were within the ranges reported for healthy juvenile gilthead seabream (Peres *et al*., 2012). Blood glucose level is regarded as hormonal response to stress factor and it is commonly used as a stress indicator (Morgan & Iwama, 2011). Feeding experimental diets did not cause variation in glucose levels in gilthead sea bream in the present investigation. Similar results were reported by Ballester-Lozano *et al*. (2015). Peres *et al.* (2012) in a previous study showed that gilthead sea bream is able to maintain glucose homeostasis after a period of starvation of <1 week and that plasma glucose content glucose is not an adequate indicator of fish nutritional status in this species. Although blood glucose content level is regarded as hormonal response to stress factor and it is commonly used as a stress indicator (Morgan & Iwama, 2011), this may not be so in gilthead sea bream, except under extended periods of starvation.

ALP, GPT and GOT are liver enzymes in blood and are critical indicators of hepatic tissue malfunction (Babalola *et al*., 2009), as they are released into the blood when the hepatic tissue is damaged. Increased blood content of these enzymes is an important indicator for necrosis in liver tissue degeneration and impaired metabolism (Kopp & Hetesa, 2000; Yu *et al*., 2010; Sathya *et al*., 2012; Ramesh *et al*., 2014). Compared to FO fed fish, feeding of CSO and CO diets in the present investigation led to significant reduction in GOT, whereas GPT levels were unaffected. ALP levels increased in fish fed with diets containing VOs except in fish fed 60CO diet. Waagbo *et al*. (1988) suggested that ALP reduction is as a result of a generally reduced metabolic activity, which correlates with signs of anaemia (Řehulka & Minařík, 2007). Hence, changes in ALP values reflect liver or bone disorders (Yu *et al*., 2010; Kashkooli *et al*., 2011).

Inclusion of dietary ingredients of plant origin has been shown to exhibit hypocholesterolemic effect in most cultured fish (Richard *et al*., 2006a, 2006b; Jordal *et al*., 2007; Peng *et al*., 2008; Romarheim *et al*., 2008; Messina *et al*., 2013; Hartviksen *et al*., 2014). Feeding trials by Ballester-Lozano *et al*. (2015) using semi-synthetic diets highlighted that FO replacements with VOs lowered triacylglycerol (TAG) and cholesterol in gilthead sea bream. Similarly, Benedito-Palos *et al*. (2016) reported a reduction in TAG and a cholesterol lowering effect that was especially evident in gilthead sea bream fed with diets with maximum replacements of fish meal (87%) and fish oil (84%). Replacement of dietary FO with CSO or CO reduced blood contents of TAG, total cholesterol and LDL-cholesterol in juvenile gilthead sea bream in the present study, with the highest reduction ($P<0.05$) recorded in fish receiving diets in which FO was totally replaced. HDL-cholesterol was increased when CSO or CO totally replaced FO in gilthead sea bream diets in the present investigation. However, HDL was reduced when gilthead sea bream was fed diets in which blend of VOs totally replaced FO (Ballester-Lozano *et al.,* 2015). Similarly, reduced HDL-cholesterol was recorded when gilthead sea bream was fed with diets with maximum replacements of FM and FO (Benedito-Palos *et al*., 2016). The reduction in blood HDL-cholesterol recorded in the previous studies in gilthead sea bream can be attributed to the dietary n-3 HUFA deficiency.

The gut is one of the target organs when evaluating the nutritional and physiological status of fish (Gisbert *et al*., 2008). Histological examination of the gut of gilthead sea bream showed increased lipid droplet accumulation, which was elevated in fish fed with diets in which dietary FO was totally replaced with CSO or CO. Lipid droplets are intracellular organelles that store neutral lipids for use as an energy source in membrane synthesis and in production of signaling lipids (Welte, 2015), and they accumulate when ingested lipids are not oxidized. The accumulation of the lipids led to nuclei and

cytoplasmic organelle displacement in the gut of gilthead sea bream fed CSO and CO diets in the present study. Increased lipid depositions have been found in tissues of several fish species when they were fed with diets high in VOs (Fountoulaki *et al*., 2009; Benítez-Dorta *et al*., 2013; Ballester-Lozano *et al*., 2015; Betancor *et al*., 2015, 2016).

Previous studies demonstrated that dietary FA composition could influence lipid metabolism in fish (Eroldoğan *et al*., 2013; Hixson and Parrish, 2014). Cells of gilthead sea bream have active fatty acid elongases and Δ-6 desaturase (Tocher and Ghioni, 1999), which are up-regulated by dietary C_{18} PUFA (Betancor *et al*., 2016; Ofori-Mensah *et al*., 2020a, 2020b). Therefore, the increased expressions of the elongases (ELOVL4, ELOVL5, and ELOVL6) and Δ-6 desaturase recorded in the gut of fish fed with diets containing camelina or chia oil in the present study are in agreement with those of the earlier ones.

SREBP1 plays a critical role in fatty acid metabolism and *de novo* lipogenesis (Horton *et al*., 2003). Betancor *et al*. (2016) reported up-regulation in SREBP1 expression in liver but not in the foregut of gilthead sea bream fed with diets containing camelina oil. However, up-regulation in SREBP1 was observed in foregut of fish fed with CO and 60CO diets in the present study. SREBP1 regulates the expression of target genes such as FADS2, HL, LPL, CPT1a and CPT1b. Up-regulation in these target genes could lead to hydrolysis and/or oxidation, which can alter the histology of internal organs through enhanced lipid deposition or utilization.

Feeding of VO-based diets led to enhanced expression of CEL, HL, LPL and HSL in the intestine. These lipases have been reported to promote lipolysis, which in turn lead to lipid deposition. For instance, up-regulation in the expression of CEL in the gut of fish fed with VO-based diets in the present investigation is consistent with a previous study in which CEL expression was up-regulated when gilthead sea bream was fed with low FO diets (Torno *et al*., 2018). LPL has been reported to mediate in the hydrolysis of triacylglycerol in lipoproteins whereas HL converts intermediate density lipoprotein to low density lipoprotein, mediating uptake of fatty acids by tissues (Lafontan & Langin, 2009; Betancor *et al*., 2016). Fat accumulation was favoured by the upregulation of hepatic LPL expression (Saera-Vila *et al*., 2005) and HSL (Cruz-Garcia *et al*., 2011) in gilthead sea bream. Thus, the up-regulation of the CEL, HL, LPL and HSL in gilthead sea bream fed with VO-based diets in the present study may in part led to lipid accumulation in the enterocytes, as reported in other studies (Cruz-Garcia *et al*., 2011; Peng *et al*., 2014; Betancor *et al*., 2016).

FAS is an important enzyme that regulates the *de novo* biosynthesis of long-chain fatty acids (Dong *et al*., 2014) and it is the main lipogenic enzyme that catalyzes successive condensation reactions to form a fatty acid (Chen *et al*., 2015). Feeding VO-based diets led to high mRNA expression in FAS in the intestines of juvenile gilthead sea bream in the present investigation. Similarly, up-regulation in FAS was recorded when dietary n-3 HUFA decreased and VO replaced FO (Jin *et al*., 2017) in juvenile black sea bream. Likewise, an up-regulation in FAS was recorded in Atlantic salmon when it was fed with VO diets (Morais *et al*., 2011). Increased deposition of glycogen and a higher lipogenic potential, indicated by FAS, glucose-6-phosphate dehydrogenase and malic enzyme activities were recorded in the liver of juvenile gilthead sea bream (Castro *et al*., 2016). Similarly, up-regulation in lipogenesis promoted lipid accumulation in enterocytes in previous studies (Caballero *et al*., 2003; Olsen *et al*., 2003; Morais *et al*., 2012). Lipogenesis was down-regulated in Atlantic salmon fed with FO diet, as demonstrated by decreased FAS expression (Morais *et al*., 2012). The increased lipid accumulation in enterocytes in juvenile gilthead sea bream recorded in the present investigation could in part be due to the increased expression of lipogenic genes.

LPCAT1 was up-regulated in the foregut of fish fed with diets containing camelina or chia oil in the present study. Betancor *et al*. (2016) reported increased LPCAT1 expression in gilthead seabream fed with diets in which FO was totally replaced with wild-type and genetically modified CSO. In a previous study, Bonacic *et al*. (2016) reported similar levels of LPCAT1 transcription in larvae of Senegalese sole (*Solea senegalensis*) fed with FO and linseed oil diets, and it was higher than that recorded in soybean oil fed larvae. These results suggest that LPCAT1 may have substrate preference for *n*-3 C_{18} PUFA, although both 18:2-acyl-CoA and 18:3-acyl-CoA are both substrates for LPCAT1 lysophosphatidylglycerol acyltransferase activity.

CPT and PPAR are vital genes that can regulate fatty acid oxidation in response to alterations in the nutrition and energy requirement in animals (Ji *et al*., 2011; Benedito-Palos *et al*., 2014). CPT1a and CPT1b genes involved in fatty acid oxidation in the mitochondria, were up-regulated in fish fed with the chia oil-based diet (CO and 60CO diets) in the present study. PPARs belong to the nuclear receptor superfamily and they play a key role in lipid metabolism, acting as specific sensors of fatty acids and cholesterol (Benedito-Palos *et al*., 2014). Feeding camelina and chia oil-based diets led to increased expression of PPARα and PPARβ genes in the present study. PPARα is a major activator of fatty acid oxidation pathways (Evans *et al*., 2004). Dietary ALA significantly increased the mRNA levels of hepatic fatty acid oxidation enzymes including CPT I and II in rats (Ide, 2000) and CPT I in Large Yellow Croaker (Qui *et al.,* 2017). The increased molecular expression of CPT and PPAR may be indicative of increased fatty acid oxidation in juvenile gilthead sea bream fed diets containing camelina and chia oil in the present study. The expression of COX2 (a mitochondrial catabolic gene) in the intestine did not differ among the dietary treatments in the present study, signifying a lack of nutritional regulation.

In conclusion, although inclusion of camelina or chia oils altered the fatty acid profile of the experimental diets, minor differences in the digestibility of individual fatty acids were recorded in the present study. Feeding CSO and CO diets to juvenile gilthead sea bream reduced plasma levels of triglyceride and cholesterol, whereas glucose levels were unaffected. Increasing inclusion levels of camelina or chia oils in the diets of gilthead seabream, elevated the expression of genes responsible for fatty acid synthesis, lipid lipolysis and lipogenesis, which fostered lipid deposition. This resulted in histological changes characterized by accumulation of lipid droplets in the fore and midguts of gilthead seabream fed with CSO and CO diets. Results of the gene expression also indicated an increased molecular expression of CPTs, PPARs and LPCAT1 in juvenile gilthead sea bream fed VO-based diets. These genes are involved in lipid oxidation and remodeling, and such an increased expression of these genes could be a compensatory mechanism to offset the increased accumulation of lipid droplets in the gut of gilthead sea bream fed with CSO or CO diets.

Ethical Statement

The Institutional Animal Care Committee of the Mediterranean Fisheries Research Production and Training Institute approved in advance all procedures (Protocol number 68385072-325.04-0967) used in this study.

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Author Contribution

Samuel Ofori-Mensah: Conceptualization, Methodology, Writing- Original draft preparation, Funding acquisition, Investigation, Data curation, Formal analysis, Project Administration. Mustafa Yıldız: Supervision, Conceptualization, Funding acquisition, Investigation, Writing- Reviewing and Edit. Vahap Eldem: Writing- Reviewing and Edit, Software, Funding acquisition. Çiğdem Ürkü: Writing- Reviewing and Edit, Visualization, Funding acquisition. Çağlayan Kaplan: Writing- Reviewing and Edit, Data curation, Formal analysis.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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