Fish Physiol Biochem (2019) 45:681–695
https://doi.org/10.1007/s10695-018-0579-9

Vegetable oil and carbohydrate-rich diets marginally affected intestine histomorphology, digestive enzymes activities, and gut microbiota of gilthead sea bream juveniles

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Received: 16 March 2018 / Accepted: 8 October 2018 / Published online: 26 October 2018
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Abstract For an increased incorporation of plant ingredients in aquafeeds at the expense of fish meal (FM) and fish oil (FO), more knowledge is needed on the effects at the intestine level of dietary vegetable oils (VO) and carbohydrates (CH), and of possible interactions. For that purpose, in this study, the activities of digestive pancreatic enzymes (amylase, lipase, total alkaline proteases), gut microbiota, and histomorphology were assessed in gilthead sea bream (IBW 71.0 ± 1.5 g) fed four diets differing in lipid source (FO or a blend of VO) and carbohydrate content (0% or 20% gelatinized starch) for 81 days. No major changes in digestive enzyme activities were noticed in fish fed the experimental diets. Dietary VO, but not CH content, modified intestinal microbial profile, by increasing the similarity of bacterial communities. Especially when combined with CH, dietary VO promoted abnormal enterocyte architecture. Liver histology was also accessed, and an increased cytoplasmic vacuolization of hepatocytes was related with dietary CH inclusion, being only significantly different in fish fed FO-based diets. Overall, nutritional interactions between dietary lipid source and carbohydrate content were not observed on digestive enzyme activities and microbial profile. However, the intestine histological modifications observed in fish fed the VOCH+ diet suggest a negative interaction between dietary VO and CH. This requires a more in depth assessment in future studies as it can have negative consequences at a functional level.

Keywords Alternative ingredients · Digesta microbial profile · Fish digestive function · Intestinal health status · Nutrition

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Introduction

The withdrawal of fish meal (FM) and fish oil (FO) by plant ingredients in carnivorous fish aquafeeds has become essential for a sustainable development of aquaculture. The shift in diet formulation from marine resources towards plant protein and vegetable oil (VO) introduces, however, new challenges such as lack of polyunsaturated fatty acids (PUFA) and unbalanced n-3/n:6 fatty acid (FA) profiles (Tocher 2015). Also, high amounts of carbohydrates, non-starch polysaccharides, antinutrients are challenges particularly relevant for marine carnivorous fish species, whose natural diets are almost devoid of those components (Enes et al. 2009; Gatlin et al. 2007; Tocher 2015). The presence of such dietary components may be responsible for negative effects on fish growth, organ functionality and

physiology, and animal's health (Francis et al. 2001; Krogdahl et al. 2010; Montero et al. 2010). To increase the use of plant protein and VO in aquafeeds is thus essential to deepen our understanding of the extent of such dietary manipulations on fish physiological functions and health. In addition, more knowledge is needed on the effects of dietary nutrients per se, such as carbohydrates and VO, as well on possible interactive effects between them. However, studies assessing the potential interactions between dietary nutrients on fish physiological functions are scarce.

Gilthead sea bream (*Sparus aurata*), one of the main marine carnivorous fish species produced in southern Europe, has been successfully grown on diets with large proportions of FM protein replaced by appropriate mixtures of plant protein sources (De Francesco et al. 2007; Gómez-Requeni et al. 2004; Kissil and Lupatsch 2004; Santigosa et al. 2008) and including up to 20% carbohydrates (Couto et al. 2008; Enes et al. 2008; Fernandez et al. 2007). VO has also successfully replaced up to 60–66% FO in diets for gilthead sea bream, but higher replacement levels are challenging due to the specific requirement of gilthead sea bream for long-chain (LC-PUFA), which are not present in VO (Benedito-Palos et al. 2008; Benedito-Palos et al. 2007; Caballero et al. 2004; Fountoulaki et al. 2009; Izquierdo et al. 2005; Izquierdo et al. 2003; Montero et al. 2010; Wassef et al. 2007).

Several studies have assessed the impact of plant ingredients on intestinal morphology and digestive enzymes activity of gilthead sea bream. For instance, plant protein included at different levels in the diets was shown to decrease villi length (Martinez-Llorens et al. 2012; Santigosa et al. 2008) and thickness (Baeza-Arino et al. 2016; Martinez-Llorens et al. 2012; Santigosa et al. 2008), as well as hypertrophy of the submucosal layer (Sitja-Bobadilla et al. 2005) of several intestine sections. In addition, lipid droplet accumulation in anterior intestine enterocytes was the most reported effect related with dietary FO replacement by VO (Caballero et al. 2003; Santigosa et al. 2011). Regarding digestive capacity, plant protein-based diets decreased intestinal trypsin (Robaina et al. 1995), total alkaline protease (Santigosa et al. 2008), alkaline phosphatase, and γ-glutamyl transpeptidase (Silva et al. 2010) activities, while no effects were recorded on amylase (Santigosa et al. 2008), aminopeptidase N, and maltase (Silva et al. 2010) activities. Replacing FO with VO in the diets had no marked effects on amylase and lipase activities, while total alkaline protease activity increased in pyloric caeca and decreased in proximal intestine (Santigosa et al. 2011).

The possible modulation of gastrointestinal microbial composition by dietary plant ingredients has also recently attracted considerable attention, due to the role played by the gastrointestinal microbiota in key functions of fish, including nutrition, intestinal integrity, and immunity (Gómez and Balcázar 2008; Nayak 2010; Ray et al. 2012; Ringo et al. 2016). Inclusion of plant ingredients in fish diets may contribute with important amounts of non-starch polysaccharides, which may be fermented by microorganisms in the intestine, thus inducing modifications in the composition of the microbiota community (Clements 1997; Gatesoupe et al. 2014; Stone 2003). On the other hand, the FA composition of VO is different from that of FO, and may induce modifications in intestinal membrane lipid composition, function, and fluidity, which may affect adherence mechanisms of microorganisms to enterocytes, lead to changes on bacterial metabolic processes, and modify the intestinal microbiota profile. Studies on the effects of dietary plant proteins on gilthead seam bream gut microbiota are somehow contradictory. While Silva et al. (2011) observed minor changes in intestinal microbiota composition related to plant protein-based diets, other studies showed that dietary inclusion of plant proteins affected the intestinal bacterial profile (Dimitroglou et al. 2010) or the relative abundance of some bacterial groups, such as Firmicutes and Proteobacteria (Estruch et al. 2015). The effects of VO on gastrointestinal microbiota remain, to our knowledge, unexplored.

Previously, we have shown that the dietary lipid source (FO or VO) affects the response of target metabolic pathways (such as cholesterol body pools, lipid and glycogen body allocation) to high-carbohydrate diets, suggesting an interactive effect between dietary VO and CH, at least at metabolic level (Castro et al. 2016b).

Thus, the aim of the current study was to assess the potential interactive effects of dietary carbohydrate and lipid sources on gilthead sea bream gut microbiota profile and digestive-related aspects (digestive enzymes and histomorphology). In addition, the histomorphology of the liver was also assessed, as it is a crucial active metabolic organ.

Material and methods

Experimental diets

Four diets were formulated differing in carbohydrate content (0 and 20% gelatinized starch, diets CH− and CH+, respectively) and lipid source (diets FO and VO) (Table 1). Carbohydrate inclusion in the diets was achieved by replacing protein, which was kept above requirements for the species in all diets (Oliva-Teles 2000). FM was added as the only dietary protein source and it was partially replaced by gelatinized starch in CH+ diets. Diets were isolipidic and the major lipid source of FO diets was cod liver oil. In VO diets, cod liver oil was replaced by a VO blend composed of 20% rapeseed, 50% linseed, and 30% palm oils.

Diets presented some differences in FA composition due to lipid sources (Table 2). The proportion of total saturated fatty acids (SFA) was similar among diets, but monounsaturated fatty acids (MUFA) were higher in FO diets, and n-3 and n-6 polyunsaturated fatty acids (n-3, n-6 PUFA) were slightly higher in VO diets. Within MUFA, higher levels of oleic acid (18:1 n-9) were recorded in VO diets, while the opposite occurred for palmitoleic acid (16:1 n-7), eicosenoic acid (20:1 n-9), and erucic acid (22:1 n-9). FO diets had higher levels of EPA and DHA than VO diets. Total n-3 and n-6 PUFA were higher in VO diets, mainly due to linolenic acid (18:3 n-3) and linoleicacid (18:2 n-6) levels, respectively. Details of diet preparation and analysis are given in Castro et al. (2016b).

Table 1 Ingredient and chemical composition of the experimental diets

	Experimental diets			
Lipid source	FO		VO	
Carbohydrates	CH−	CH+	CH−	CH+
Ingredients (% dry weight)				
Fish meal[a]	87.3	65.1	87.3	65.1
Starch[b]	0	20	0	20
Cod liver oil[c]	9.2	11.4	0	0
Vegetable oil blend[d]	0	0	9.2	11.4
Vitamins[e]	1.5	1.5	1.5	1.5
Minerals[f]	1.0	1.0	1.0	1.0
Binder[g]	1.0	1.0	1.0	1.0
Proximate analyses (% dry matter)				
Dry matter (DM)	87.0	86.8	87.2	87.6
Crude protein (CP)	66.3	50.3	66.3	50.4
Crude fat (CF)	18.4	18.4	18.2	18.3
Starch	–	16.8	–	18.0
Energy	22.7	23.3	23.3	22.7
Ash	14.1	11.2	14.3	11.1

Fish oil (FO), blend of vegetable oils (VO), carbohydrate content 0% (CH−) or 20% (CH+) gelatinized maize starch

[a] Steam-dried LT fish meal (Superprime). Inproquisa, Madrid, Spain (CP 74.6% DM; CL 10.1% DM)

[b] C-Gel Instant-12018. Cerestar, Mechelen, Belgium

[c] Labchem, Laborspirit Lda., Lisboa, Portugal

[d] 30% palm oil (Colmi. Malasia). 50% linseed oil (Sociedade Portuense de Drogas, S.A., Portugal) and 20% rapeseed oil (Huilerie Emile Noël S.A.S., France)

[e] Vitamins (mg kg^{-1} diet): retinol acetate, 18,000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400. Premix, Viana do Castelo, Portugal

[f] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet). Premix, Viana do Castelo, Portugal

[g] Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate) Agil, England

Animals, experimental conditions, and sampling

The experiment was directed by accredited scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The study was performed at the Marine Zoological Station, University of Porto, Portugal, in a thermoregulated recirculation water system equipped with 12 fiberglass cylindrical tanks with a water capacity of 300 L and supplied with a continuous flow of filtered seawater. Gilthead sea bream (*Sparus aurata*) juveniles were adapted to the experimental conditions for 2 weeks. Thereafter, 12 groups of 21 juveniles with an initial body weight of 71.0 ± 1.5 g were established, and the experimental diets were randomly assigned to triplicate groups of these fish. During the trial, salinity averaged 34.7 ± 0.8 g L^{-1}, dissolved oxygen was kept near saturation, and water temperature was regulated to 24.0 ± 0.5 °C. The growth trial lasted 81 days and during this period, fish were hand-fed twice a day, 6 days a

Table 2 Fatty acid composition (% of total fatty acids) of the experimental diets

	Experimental diets			
Lipid source	FO		VO	
Carbohydrates	CH−	CH+	CH−	CH+
14:00	5.8	5.9	2.5	2.0
15:00	0.7	0.6	0.4	0.3
16:00	18.7	17.2	21.3	21.0
17:00	0.5	0.4	0.4	0.3
18:00	4.0	3.5	4.9	4.5
20:00	0.2	0.1	0.3	0.2
∑SFA	29.9	27.8	29.9	28.6
16:1n-7	7.1	7.5	2.3	1.8
18:1n-9	18.3	19.1	25.4	27.6
20:1n-9	4.9	5.8	0.9	0.7
22:1 n-9	3.9	4.3	0.5	0.4
∑MUFA	34.4	36.8	29.2	30.5
18:2 n-6	2.2	2.2	9.0	10.6
18:3 n-6	0.1	0.1	0.0	0.1
20:2 n-6	0.3	0.3	0.1	0.1
20:3 n-6	0.13	0.12	0.08	0.08
20:4 n-6	1.2	1.0	0.9	0.7
∑n-6 PUFA	4.0	3.8	10.4	11.6
18:3 n-3	1.1	1.2	15.7	19.0
18:4 n-3	1.9	2.1	0.5	0.4
20:3 n-3	0.14	0.15	0.06	0.04
20:4 n-3	0.6	0.6	0.2	0.1
20:5 n-3	7.8	8.0	3.5	2.5
21:5 n-3	0.3	0.3	0.1	0.1
22:5 n-3	1.2	1.2	0.7	0.5
22:6 n-3	11.7	10.8	7.2	4.9
∑n-3 PUFA	24.8	24.3	28.0	27.5
Ratios				
Sat/PUFA	1.0	0.9	0.8	0.7
n-3/n-6	6.2	6.4	2.7	2.4
Insat. Index	181.4	179.5	169.2	160.2

Dietary lipid source (LS), fish oil (FO), blend of vegetable oils (VO), carbohydrate content: 0% (CH−) or 20% (CH+) gelatinized maize starch

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *n-3 LC-PUFA* n-3 long-chain polyunsaturated fatty acids, *Insat. Index* insaturation index

week, to apparent visual satiety. At the end of the trial, fish were unfed for 1 day to empty gut content and then bulk-weighed after mild anesthesia with 0.3 mL/L methylethanol (VWR Chemicals, Alfragide, Portugal). To reduce handling stress, fish were fed for 3 additional days and then, 6 h after the last meal, five fish from each tank were randomly sampled and euthanized by a sharp blow to the head.

Three fish per tank were dissected on chilled trays, and the liver and digestive tract were excised and freed from adherent adipose and connective tissues. For histological and digestive enzyme analyses, the intestine was divided in anterior intestine (AI, section between the last pyloric caeca and the mid-line of the intestinal length) and posterior intestine (PI, section from the mid-line of the intestinal to distal intestine). Circa 0.5 cm of the liver, first part of AI, and distal intestine (DI, visually distinguished by a darker and thicker mucosa) were collected for histological evaluation. The liver and intestine samples were rinsed in phosphate-buffered saline (PBS), blotted dry with a paper towel, immediately fixed in phosphate-buffered formalin (4%, pH 7.4) for 24 h, and then transferred to ethanol (70%) until further processing. For digestive enzyme analyses, each AI and PI sections with intestinal content were immediately frozen in liquid nitrogen and stored at − 80 °C until analysis.

Two other fish per tank were sampled under aseptic conditions and pooled for characterization of microbiota. Digesta was collected by squeezing the entire intestine, immediately frozen in liquid nitrogen, and stored at − 80 °C until analysis.

Digestive enzyme activities

Digestive tract sections were homogenized (dilution 1:6, *w*/*v*) in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA, and 0.1%Triton-X-100 (*v*/*v*), pH 7.8) and centrifuged at 30,000*g* for 30 min at 4 °C. The resultant supernatants were collected, and aliquots were stored at − 80 °C until digestive enzymes analysis.

For each enzyme activity, assay dilution tests were previously done to ensure optimum ratio between enzyme and substrate. All enzyme activities were measured at 37 °C in a Multiskan GO microplate reader (model 5111 9200; Thermo Scientific, Nanjing, China). The specific assay conditions for each enzyme were as follows:

α-Amylase (EC 3.2.1.1) activity was determined with a commercial kit (ref. 41201, Spinreact, Girona, Spain), with modification in the proportion of supernatants and assay buffer (200 μL of assay buffer with 10 μL of supernatants). The rate of product formation

(2-chloro-4-nitrophenol) was quantified at 405 nm. Lipase (EC 3.1.1.3) activity was determined using a commercial kit (ref. 1001275, Spinreact, Girona, Spain) with modification in the proportion of supernatants and assay reactives (200 μL of the assay buffer, 40 μL substrate with 10 μL of supernatants). 1-2-O-dilaurylrac-glycero-3-glutaric acid-(60 methylresorufin)-ester was used as substrate, and the formation rate of methylresorufin was followed at 580 nm. Total alkaline protease activity was determined by the casein-hydrolysis method described by Walter (1984) and adapted by Hidalgo et al. (1999). A reaction mixture containing 0.25 mL casein at 1% (*w*/*v*), 0.25 mL buffer (0.1 M Tris–HCl, pH 9), and supernatant from the homogenates (0.1 mL) was incubated for 1 h at 37 °C. A control blank for each sample was assayed, adding the supernatant from the homogenates after incubation time. The reaction was stopped by addition of 0.6 mL 8% (*w*/*v*) trichloroacetic acid solution to the blanks and reaction samples. After being kept for 1 h at 4 °C, blanks and reaction samples were centrifuged at 1800*g* for 10 min and the absorbance of supernatants measured at 280 nm. Tyrosine solution was used as standard.

Unit (U) of enzyme activity was defined as micromole (μmol) of product generated per minute under the measurement conditions described above and expressed per milligram soluble protein (specific activity). Protein concentration was determined according to Bradford (1976) using a SigmaAldrich (Química, S.L., Sintra, Portugal) protein assay kit (ref. B6916) with bovine serum albumin as standard.

Histology

Histological samples were processed and sectioned using standard histological techniques and stained with hematoxylin and eosin. Blinded evaluation was performed using a semi-quantitative scoring system ranging from 1 to 5. Score 1 was considered the normal tissue appearance and subsequent scores accounted for increasing alterations towards normal tissue histomorphology. Intestinal samples were evaluated according to the criteria suggested by Krogdahl et al. (2003): widening and shortening of intestinal folds, increased cellularity of connective tissue and widening of lamina propria and submucosa, infiltration of mixed leucocyte population (namely intraepithelial lymphocytes and eosinophilic granular cells) in the lamina propria and submucosa, nucleus position within the enterocytes, loss of supranuclear vacuolization in the absorptive cells (enterocytes) of the intestinal epithelium. The liver samples were evaluated for general histomorphology, giving particular attention to cytoplasm vacuolization of hepatocytes and any signs of inflammation.

Intestinal microbiota analysis

DNA extraction from digesta samples

DNA was extracted from a pool of two fish per tank to reduce variation, and extraction was performed according to Pitcher et al. (1989) with some modifications. Briefly, approximately 300 mg of intestinal contents were resuspended in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) vigorously mixed and pelleted by centrifugation at 13,000*g* for 5 min. After two washes with 1 mL TE, cell pellet was resuspended in 200 μL of TE containing 50 mg/mL of lysozyme and incubated for 30 min at 37 °C. A second 30-min incubation at 37 °C was performed with the addition of 10 mg/mL RNAse, followed by a 30-min incubation at 55 °C with 20 mg/mL Proteinase K and 10% SDS. After 10 min on ice in the presence of 500 μL of GES (Pitcher et al. 1989) and 250 μL of ammonium acetate (7.5 M), a phenol-chloroform extraction was performed by adding 500 μL phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was re-extracted with 500 μL of chloroform-isoamyl alcohol (24:1) and DNA of the subsequent aqueous phase was precipitated with 0.6 volumes of isopropanol. After 10-min centrifugation at 13,000*g*, the DNA pellet was washed with ice-cold 70% ethanol and dried at room temperature. DNA was resuspended in 50 μL ultrapure water.

Polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis DGGE

Bacterial 16S rRNA gene fragments were amplified by touchdown PCR on a T100™ Thermal Cycler (Bio-Rad Laboratories Lda., Amadora, Portugal), using primers 16S-358F (which has a GC clamp at the 5′end) and 16S-517R (Muyzer et al. 1993), yielding a 233 bp DNA fragment. PCR mixtures (50 μL) contained 24.75 μL of water (Sigma, Sintra, Portugal), 10 μL of GoTaq Buffer 5X (PROMEGA, Carnaxide, Portugal), 5 μL of each dNTPs (2 mM, PROMEGA, Carnaxide, Portugal), 2.5 μL of each primer (10 μM Forward and Reverse), 0.25 μL of GoTaq polymerase (PROMEGA,

Carnaxide, Portugal), and 5 μL of DNA template were subjected to touchdown PCR. A 94 °C incubation for 5 min was followed by 10 cycles of 64 °C, 1 min, 65 °C, 1 min, and 72 °C, 3 min. The annealing temperature was decreased at every cycle 1 °C, until reach 55 °C. Thus, final 20 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min. Final extension was at 72 °C, 10 min. PCR products were resolved by electrophoresis on 1% (*w*/*v*) agarose gels containing Gel Red (Biotium) to check for product size. DNA concentration was quantified with the μDrop™ Plate (Thermo Fisher Scientific Inc., Nanjing, China) and 300 ng of each PCR product was loaded on 8% polyacrylamide gel composed by a denaturing gradient of 40 to 80% 7 M urea/40%formamide. Electrophoresis occurred on a DCode™ universal mutation detection system (Bio-Rad Laboratories Lda., Amadora, Portugal), during 16 h at 60 °C, 65 V in 1 × TAE buffer. Gels were stained for 1 h with SYBR-Gold Nucleic Acid Gel Stain and imaged on a Gel Doc EZ System (Bio-Rad Laboratories Lda., Amadora, Portugal) with the Image Lab software v4.0.1 (Bio-Rad Laboratories Lda., Amadora, Portugal). Selected bands were excised from the gel and eluted in 20 μL ultrapure water prior to DNA reamplification using the same oligonucleotide primers as above, but without the GC clamp. Amplicons were sequenced to identify microbiota OTUs (Operational Taxonomic Units). Phylogenetic analysis, to identify the closest known species, was done by comparison with sequences in the GenBank non-redundant nucleotide database using BLAST (http://www.ncbi.nlm.nih.gov). Only sequences higher than 100 bp reads and 80–100% query coverage were considered a valid identification.

Statistical analysis

Digestive enzyme activity data were checked for normal distribution and homogeneity of variances (Shapiro–Wilk and Levene tests, respectively) and normalized when appropriate. Statistical evaluation of data was carried out by a 2 × 2 factorial arrangement of treatments in a completely randomized design (two-way ANOVA) with carbohydrate level and lipid source as fixed factors. Histological data was not normal nor homogeneous (Shapiro–Wilk and Levene tests, respectively) and could not be normalized; thus, the non-parametric Kruskal–Wallis with multiple comparisons test was performed. DGGE banding patterns were transformed into presence/absence matrices and band intensities measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories, Lda., Amadora, Portugal). Relative similarities between dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd., Ivybridge, UK). Similarity percentages (SIMPER) were used to represent the relative similarities between treatments. Species richness was assessed using Margalef's measure of richness, and species diversity was assessed by the Shannon–Weaver index. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA). Parameters were subjected to two-way ANOVA, with lipid source and carbohydrate level as fixed factors. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were done using the IBM SPSS 23 software package (SPSS Inc., Chicago, IL, USA) for Windows.

Results

Fish promptly accepted the experimental diets and no mortality occurred during the trial. Results of the feeding trial were not the aim of this study and were presented elsewhere (Castro et al. 2016b). In short, no differences in growth performance and feed efficiency were observed due to diet composition. Final body mass and feed efficiency averaged 215.0 g and 0.7 respectively.

Dietary VO or carbohydrate inclusion did not affect lipase, total alkaline protease, and amylase activities in both AI and PI of gilthead sea bream (Table 3). Nevertheless, lipase activity in AI was almost significantly decreased ($p = 0.05$) by the dietary incorporation of carbohydrates.

With the exception of SM in AI, intestine histomorphology was not affected by dietary CH within dietary lipid source (Table 4). FOCH− diet induced increased cellularity of AI submucosa than FOCH+ diet. In contrast, VO inclusion in the diets resulted in intestinal histomorphological alterations, which were significant in fish fed the CH+ diets (Table 4). VOCH+ showed higher mean scores than its FO counterpart, mainly due to increased width, cellularity of lamina propria and submucosa (LP, SM), and number of eosinophilic granular cells (EGCs) throughout the intestine and due to abnormal enterocyte architecture, with loss of the typical supranuclear vacuolization (SNV) in the AI (Table 4, Fig. 1).

Table 3 Specific digestive enzyme activities (mU mg^{-1} protein) in the anterior (AI) and posterior (PI) intestine of gilthead sea bream fed the experimental diets

Diets	FO		VO		p value*		
	CH−	CH+	CH−	CH+	LS	CH	LS × CH
Lipase							
AI	4.38 ± 2.38	2.70 ± 0.95	3.98 ± 2.18	2.88 ± 1.27	0.866	0.050	0.619
PI	5.91 ± 3.15	6.76 ± 2.76	7.38 ± 4.78	8.50 ± 4.60	0.232	0.458	0.919
TAP							
AI	126.6 ± 32.1	119.9 ± 27.4	116.7 ± 44.4	115.8 ± 23.6	0.535	0.732	0.794
PI	132.4 ± 36.2	151.04 ± 39.5	120.10 ± 29.8	122.3 ± 27.8	0.088	0.379	0.484
α-Amylase							
AI	170.7 ± 106.2	98.7 ± 30.6	125.6 ± 66.6	136.3 ± 46.2	0.988	0.486	0.078
PI	150.6 ± 87.4	208.8 ± 100.9	171.9 ± 83.5	205.4 ± 107.4	0.783	0.167	0.706

Dietary lipid source (LS), fish oil (FO), blend of vegetable oils (VO), carbohydrate content: 0% (CH−) or 20% (CH+) gelatinized maize starch

Total alkaline proteases (TAP)

Values are presented as means ± standard deviation (SD), $n = 9$

* Significant differences at $p < 0.05$ two-way ANOVA

Table 4 Details of the score-based evaluation of liver and intestinal histology (anterior intestine, AI, and distal intestine, DI) of gilthead sea bream fed the experimental diets by the end of the trial, based on changes observed in cytoplasm vacuolization of hepatocytes and any signs of inflammation for liver samples: mucosal fold heights (shortening, widening and fusion of intestinal folds, FH), connective tissue hyperplasia in the lamina propria (LP) and in the submucosa (SM), number of intraepithelial lymphocytes (IELs), eosinophilic granular cells (EGCs), nucleus position within the enterocytes (ENT), and variation of supranuclear absorptive vacuolization (SNV) in intestinal sections. An overall score (mean) was calculated by averaging the scores of the individual characteristics evaluated

Section	Parameter	FOCH−	FOCH+	VOCH−	VOCH+	p value*
AI	FH	1.1 ± 0.33	1.1 ± 0.33	1.6 ± 0.73	1.8 ± 0.83	0.071
	LP	1.9 ± 0.33^{ab}	1.1 ± 0.33^{a}	2.0 ± 1.00^{ab}	2.7 ± 0.71^{b}	< 0.001
	SM	1.9 ± 0.33^{b}	1.1 ± 0.33^{a}	1.6 ± 0.53^{ab}	1.9 ± 0.33^{b}	0.002
	IELs	2.1 ± 0.33	1.6 ± 0.88	2.1 ± 1.05	2.1 ± 0.93	0.303
	EGCs	2.0 ± 0.50^{ab}	1.3 ± 0.71^{a}	2.6 ± 0.53^{b}	2.4 ± 1.01^{b}	0.007
	ENT	1.1 ± 0.33^{a}	1.4 ± 0.53^{ab}	2.1 ± 0.93^{b}	2.0 ± 0.71^{b}	0.016
	SNV	1.1 ± 0.33^{a}	1.4 ± 0.73^{a}	1.9 ± 0.93^{ab}	2.4 ± 0.53^{b}	0.002
	Mean	1.6 ± 0.12^{ab}	1.3 ± 0.32^{a}	2.0 ± 0.33^{bc}	2.2 ± 0.38^{c}	< 0.001
DI	FH	1.0 ± 0.00^{a}	1.8 ± 0.67^{ab}	2.0 ± 1.00^{b}	2.1 ± 1.05^{b}	0.014
	LP	1.0 ± 0.00^{a}	1.0 ± 0.00^{a}	1.3 ± 0.50^{ab}	2.0 ± 0.50^{b}	< 0.001
	SM	1.2 ± 0.44	1.2 ± 0.44	1.1 ± 0.33	1.0 ± 0.00	0.478
	IELs	2.2 ± 0.67	1.7 ± 1.12	2.1 ± 0.78	2.1 ± 0.33	0.246
	EGCs	1.9 ± 0.93^{ab}	1.4 ± 0.53^{a}	2.0 ± 0.87^{ab}	3.1 ± 0.78^{b}	0.004
	ENT	1.2 ± 0.67	1.7 ± 0.71	1.4 ± 0.73	1.9 ± 0.60	0.090
	SNV	1.2 ± 0.67	1.6 ± 0.73	1.7 ± 0.87	2.0 ± 0.71	0.110
	Mean	1.4 ± 0.36^{a}	1.5 ± 0.29^{a}	1.7 ± 0.29^{ab}	2.0 ± 0.33^{b}	0.004
Liver		1.4 ± 0.15^{a}	2.1 ± 0.44^{b}	1.5 ± 0.24^{a}	1.7 ± 0.44^{ab}	0.003

Fish oil (FO), blend of vegetable oils (VO), carbohydrate content: 0% (CH−) or 20% (CH+) gelatinized maize starch

Values are presented as means ± standard deviation (SD), $n = 9$. Score from 0 to 5, with 5 indicating major alterations

* Different lowercase letters stand for statistical differences across dietary groups as determined by the Kruskal–Wallis all pairwise comparisons ($p < 0.05$)

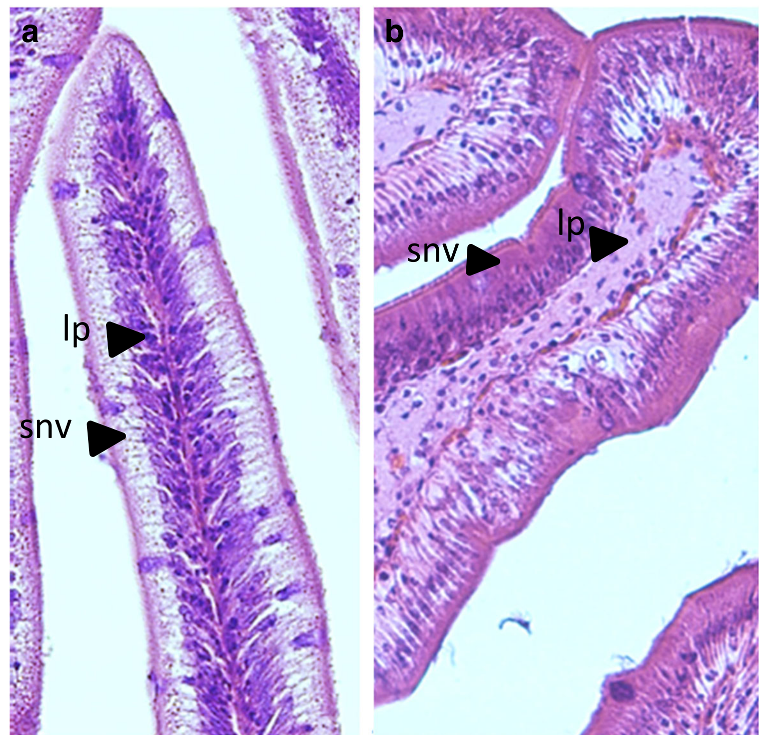

Fig. 1 Detail of gilthead sea bream DI fed FOCH+ diet (**a**) and VOCH− diet (**b**). lp: lamina propria, arrow heads: enterocytes nucleus, snv: supranuclear absorptive vacuoles. H-E staining

Liver histomorphology was affected by dietary carbohydrate level, but this effect was only significant in fish fed the FO-based diets (Table 4). The liver of fish fed the FOCH+ diet showed increased intracellular vacuolization, while CH− groups showed similar sized hepatocytes, optically empty in appearance (Fig. 2).

The Bray–Curtis dendrogram (Fig. 3) revealed two distinct clusters of intestinal microbiota, corresponding to samples recovered from fish fed the VO and FO-based diets. Within these, clustering was also observed between samples from the CH+ or CH− groups. Similar banding patterns from the three replicates for each diet condition were evident in most cases. The figure further shows that bacterial communities obtained from fish fed the VO diets were more closely related (similarity between 50 and 80%) than those recovered from fish fed the FO diets, which seem to diverge more (similarity between 50 and 70%). Despite this, average number of OTUs, microbial richness, and diversity indices were not significantly affected by diet composition (Table 5).

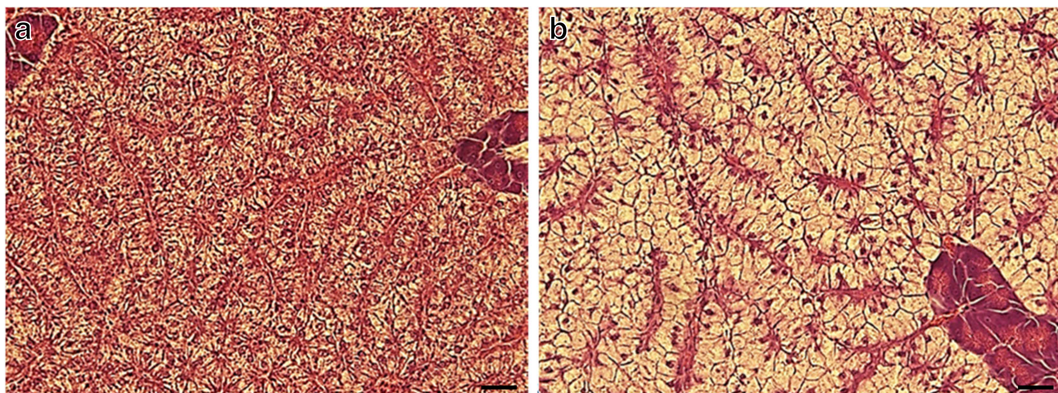

Fig. 2 The liver of gilthead sea bream fed FOCH− diet (**a**) and FOCH+ diet (**b**). Scale bar A and B: 50 μm; H-E staining

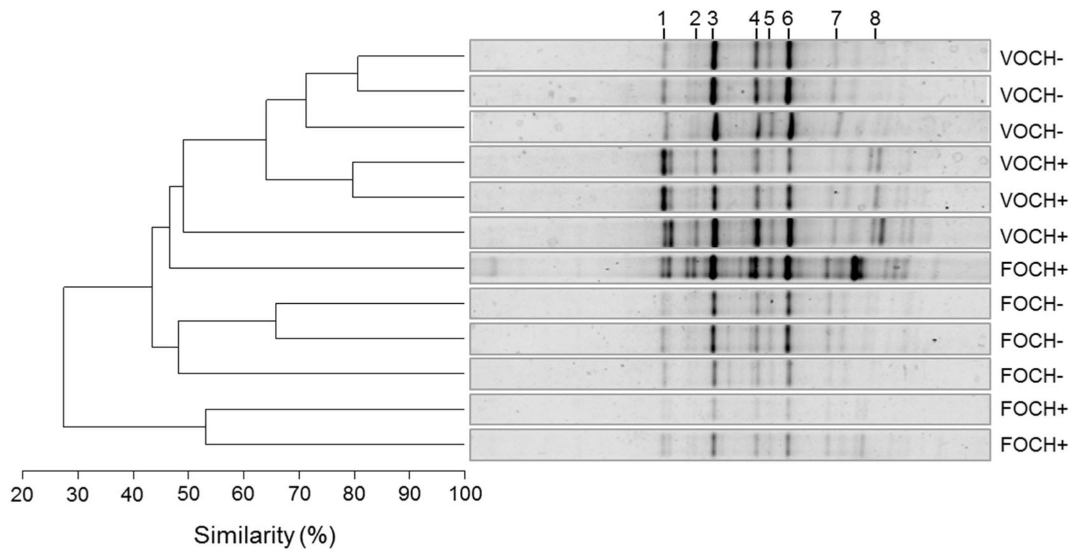

Fig. 3 Dendrograms and PCR-DGGE fingerprints of the microbiota found in digesta samples recovered from the intestines of gilthead seabream fed the experimental diets (fish oil (FO), blend of vegetable oils (VO), carbohydrate content: 0% (CH−) or 20% (CH+) gelatinized maize starch). Numbers (1–8) on top the Figure correspond to the gel bands sequenced to identify the corresponding bacterial species, described on Table 6

Nevertheless, SIMPER similarity indices were significantly higher in fish fed the VO diets compared to fish fed the FO diets (Table 5). Sequence analysis from selected DGGE bands (Fig. 3, Table 6) showed that detectable dominant bacteria present in the intestines of gilthead seabream under our experimental conditions were most closely related to the *Enterococcus*, *Bacillus*, *Pseudomonas*, *Cronobacter*, *Burkholderia*, *Corynebacterium*, and *Bifidobacterium* genera.

Discussion

Previously, we reported that gilthead sea bream growth performance was not affected by diets in which FO were replaced by VO blends up to 60% or by the inclusion of 20%CH, and that there were no interactive effects between these dietary components (Castro et al. 2016b). The current study aimed to exploit further the potential effects of such diets at the digestive level, namely on

Table 5 Ecological parameters obtained from PCR-DGGE fingerprints of the microbiota found in fecal samples recovered from the intestines of gilthead seabream fed the experimental diets

Experimental diets	FO		VO		p value*		
	CH−	CH+	CH−	CH+	LS	CH	LS × CH
OTUs[1]	11.7 ± 1.2	10.3 ± 7.8	9.7 ± 1.5	13.3 ± 3.2	0.846	0.652	0.345
Richness[2]	0.7 ± 0.1	0.6 ± 0.5	0.6 ± 0.1	0.8 ± 0.2	0.938	0.696	0.316
Diversity[3]	2.2 ± 0.3	1.6 ± 0.5	2.0 ± 0.2	2.3 ± 0.3	0.229	0.535	0.068
SIMPER Similarity (%)[4]	54 ± 13.5	30.6 ± 19.6	74.4 ± 5.6	59.6 ± 19.9	0.027	0.070	0.648

Dietary lipid source (LS), fish oil (FO), blend of vegetable oils (VO), carbohydrate content: 0% (CH−) or 20% (CH+) gelatinized maize starch

Values are presented as means ± standard deviation (D) ($n = 3$/treatment pooled from six fish)

[1] OTUs: average number of operational taxonomic units

[2] Margalef species richness: $d = (S - 1) / \log(N)$

[3] Shannons diversity index: $H' = -\sum(\text{pi}(\ln \text{pi}))$

[4] SIMPER, similarity percentage within group replicates

[*] Significant differences at $p < 0.05$ two-way ANOVA

Table 6 Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands of the intestinal communities of gilthead seabream fed the experimental diets

Band	Nearest neighbor		
	Name	Similarity (%)	Accession number
1	*Enterococcus saccharolyticus*	88	KT261205.1
2	*Bacillus pumilus*	81	GQ487539.1
3	Uncultured *Pseudomonas* sp.	100	FJ868263.1
4	*Cronobacter* sp.	81	GQ922069.1
5	Uncultured soil bacterium clone	78	EU401883.1
6	*Burkholderia bryophila*	98	HE610592.1
7	*Corynebacterium ulcerans*	97	CP010818.1
8	*Bifidobacterium asteroides*	98	KR632507.1

tissue histomorphology, digestive enzyme activities, and microbial profile.

Effects of dietary CH content

In carnivorous fish species, such as gilthead sea bream, the use of CH-rich diets has been associated to increased glycogen, liver lipid deposition, and hepatosomatic index, which were related to the relatively limited tolerance of carnivorous fish to CH (Castro et al. 2016b; Castro et al. 2015b; Kamalam et al. 2012). In the present study, the increased hepatocytes cytoplasmic vacuolization, reflecting excess accumulation of glycogen or lipids, in fish fed CH-rich diets is in agreement with the metabolic response to CH-rich diets. Although no specific staining was applied to confirm the nature (glycogen or lipids) of hepatocyte cytoplasmic vacuolization, the higher liver lipid (only in VOCH+ group) and glycogen contents recorded in fish fed the CH+ diets (Castro et al. 2016b) support that assumption. Liver histological changes resulting from high CH intake have also been reported in other species such as Siberian sturgeon (*Acipenser baeri*) juveniles (Kaushik et al. 1989), *Labeo rohita* fries (Mohapatra et al. 2003), and Wuchang bream (*Megalobrama amblycephala*) juveniles (Zhou et al. 2013). Nevertheless, the relatively low hepatic somatic index (< 2) recorded in our fish (Castro et al. 2016b) associated with the low degree of liver histomorphological alterations suggests that the altered histological condition may be readily reversible rather than be associated with a pathological condition. Evidence of dietary CH-mediated effects on intestinal morphology that could affect fish digestion process was not observed in the present study.

Despite its carnivorous nature, high-starch digestibility coefficients have been observed in gilthead sea bream fed extruded and gelatinized starch-rich diets (Castro et al. 2016a; Couto et al. 2012; Fernandez et al. 2007; Venou et al. 2003). Nonetheless, the capacity to modulate amylase activity according to dietary CH levels seems to be limited, as confirmed in the present study and on previous studies with this species (Couto et al. 2012). Also, although dietary CH was suggested to modulate other digestive enzyme activities (such as lipase or total alkaline protease) in other fish species (Keshavanath et al. 2002; Pérez-Jiménez et al. 2009), in the present study, such effect was not observed.

In this study, dietary gelatinized starch was also ineffective in modulating intestinal microbial community. Although bacteria that are reported to have the ability of producing a wide range of exogenous digestive enzymes (e.g. amylase, lipase, protease) were detected in the present study, namely *Bacillus pumilus*, *Pseudomonas sp.*, and bacteria belonging to *Enterobacteriaceae* (Ray et al. 2012), their presence apparently had no effect on the activity of digestive enzymes, as mentioned above. Though amylolytic bacteria have been mainly isolated from the gastrointestinal tract of herbivorous fish (Ray et al. 2012), the presence of an intestinal microbiota with the potential to ferment CH was also reported in carnivorous species, such as European sea bass (Leenhouwers et al. 2008). The observed lack of microbiota modulation in fish fed the present experimental diets may be partially related to the dietary CH source used. In contrast to other CH sources, such as resistant starch and non-starch polysaccharides, that are poorly or not digested, gelatinized starch is highly digestible by gilthead sea bream (Castro et al. 2016a) thus not providing substantial substract for microbial fermentation and development. Gatesoupe et al. (2014) noticed that the bacterial profile of fecal community in European sea bass fed resistant starch and lupin meal (rich in NSP) was more affected than that of fish fed highly digestible starch, namely waxy maize. Also in Nile tilapia (*Oreochromis niloticus*), a native-starch-based diet resulted in lower nutrients digestibility and higher volatile FA production in digesta (an indicator for

intestine fermentation) compared to a gelatinized-starch-based diet (Amirkolaie et al. 2006).

Although both studies seem to support our assumption on the influence of dietary CH source on intestinal microbiota, the limitations of the method adopted in this study for bacteria detection should also be kept in mind. Although PCR-DGGE (Denaturing Gradient Gel Electrophoresis) is a very useful approach to detect and identify microbiota in fish intestine without requiring a cultivation step, it only detects the most predominant taxa; thus, lacking detection sensitivity when subtle changes in OTUs abundance occurs. To overcome this technical limitation, future studies should consider higher resolution methods such as next-generation sequencing.

Effects of dietary lipid source

The potential of VO to affect normal intestine and liver histomorphology was already observed in gilthead sea bream and other species (Caballero et al. 2003; Caballero et al. 2002; Castro et al. 2016c; Fountoulaki et al. 2009; Kjaer et al. 2008; Kowalska et al. 2010; Moldal et al. 2014; Olsen et al. 2003; Olsen et al. 1999; Santigosa et al. 2011). In most of these studies, increased lipid droplet accumulation, without substantial cellular pathological damages, was reported both in hepatocytes and enterocytes of fish fed VO-based diets (Caballero et al. 2003; Caballero et al. 2002; Castro et al. 2016c; Fountoulaki et al. 2009; Kjaer et al. 2008; Kowalska et al. 2010; Olsen et al. 2003; Santigosa et al. 2011). In other studies, however, this lipid accumulation was correlated with extensive enterocytes damage (Olsen et al. 1999). In the present study, no substantial morphological changes at intestinal level were observed, but the widening and cellularity of the lamina propria with probable presence of edema suggest that fish fed VO-based diets might have developed an allergic reaction. Similar effects have been ascribed to a variety of antinutrients, including phytosterols, which are usually present in VO (Chikwati et al. 2012; Couto et al. 2014; Knudsen et al. 2008). In a recent study with gilthead sea bream, Couto et al. (2014) suggested that phytosterols may have contributed to the onset of an intestinal inflammatory response, as increased number of leukocytes in pyloric intestine was observed in fish fed a FM-based diet with 5 g kg^{-1} of phytosterols. According to the authors, as phytosterols are poorly absorbed into the bloodstream, its accumulation in the intestine may be recognized as foreign substances by the enterocytes, triggering an immune reaction.

Besides morphological changes with suspected antigenic etiology of dietary origin, negative effects on nutrients digestibility and digestive enzyme activities, mainly of the brush border enzymes, have also been observed in carnivorous fish fed plant ingredient-rich diets (Aslaksen et al. 2007; Gu et al. 2016; Krogdahl et al. 2003; Zhang et al. 2012). In addition, the distinctive dietary FA profile of VO-based diets compared to FO-based diets, as a consequence of the different chemical and physical properties of the oils (e.g. degree of unsaturation, chain length or melting point of the FA), was also referred to be involved in the modulation of digestive function (Caballero et al. 2002; Castro et al. 2015a; Geurden et al. 2009; Jutfelt et al. 2007; Koven et al. 1994; Santigosa et al. 2011). In a recent study with gilthead sea bream fed diets with VO replacing FO, it was observed that lipid digestibility decreased (Castro et al. 2016a), and that may possibly be due, among other factors (Borey et al. 2016), to a decrease of lipase activity, while in the present study, lipase activity was not modulated by dietary lipid source. Both in the present study and in that of Castro et al. (2016a), diet composition did not affect total alkaline protease and amylase activities, or dry matter, protein and starch digestibility, respectively.

The lack of dietary effect on lipase activity observed in the present study is in agreement with the results of an earlier study also with gilthead sea bream, where the effects of FO replacement by a VO blend were tested in a plant protein-rich diet (Santigosa et al. 2011). That VO blend promoted a decrease in total alkaline protease activity in the proximal intestine, which may suggest a possible interactive effect of dietary VO and plant proteins. Evaluation of other physiological indicators, namely the activity of digestive brush border enzymes, may help to ascertain if the alterations at tissue level caused by VO observed in the present study may lead to an intestinal dysfunction.

In the present study, replacement of FO by VO in the diet did not induce changes on intestinal microbial diversity or richness. The lack of effects on bacterial diversity may be considered beneficial, since reduction in diversity may facilitate pathological episodes by improving the competitive advantage of opportunistic or invading pathogens (Heiman and Greenway 2016; Nayak 2010). Conversely, two clear microbial profiles, corresponding to FO and VO groups, were

distinguished by Bray–Curtis cluster analysis, with FO causing the maximum dissimilarity in bacterial profiles. The reason for this discrepancy between groups could not be elucidated, but the observed effects might have been determined primarily by the atypical banding pattern of one of the replicates of FO group, rather than being due to diet-related effects on the microbiota. Knowledge on how dietary lipid source affects intestinal microbiota in fish is scarce.

Therefore, a deeper research of this topic is needed, particularly considering that in mammals, dietary lipid sources were shown to induce certain microbial population profiles that are normally linked with intestine inflammatory injuries or diseases (Ghosh et al. 2013; Yu et al. 2014).

Dietary CH content and lipid source interaction

The results of this study indicate that there were no interactions between dietary CH and VO on digestive enzyme activity and microbial profile. However, effects of diet composition on the liver and intestine histomorphology seemed to be nutrient interaction-related. For instance, intake of starch when coupled with dietary VO seemed to enhance intestine histomorphological effects due to dietary VO; while the histomorphological alterations on the liver induced by CH intake were more evident when fish were fed a FO-based diet.

As previously mentioned, the physiological mechanisms underlying the increased hepatocytes cytoplasmic vacuolization in fish fed CH-rich diets are normally related to the stimulatory effect of CH on lipogenesis and glycogenesis. Liver glycogen levels were high in fish fed the CH-rich diets either with FO and VO lipid sources, but increased liver lipid levels were only noticed when fed the diet with VO as lipid source (Castro et al. 2016b). This suggests that the histological observations seen in fish fed the FOCH+ diet are more likely related with the increased accumulation of glycogen rather than lipids.

On the other hand, although available data is limited, it may be speculated that the effect on the intestine histomorphology of VO in the CH-based diet was higher due to an effect of dietary CH on the time course of the digestion and absorption dynamics. Indeed, inclusion of gelatinized starch in the diets for gilthead sea bream was previously reported to modulate transcriptional mechanisms involved in lipid absorption and transport at intestinal level, probably delaying lipid digestion or absorption (Castro et al. 2016a). Similarly, in this study, gelatinized starch might have led to a slower passage of the dietary bolus along the intestine, thereby increasing the exposure time to VO allergens in the intestine.

Conclusion

Although no interactions between dietary carbohydrates and VO were noticed on digestive enzyme activity and microbial profile, the atypical intestinal histomorphology especially observed in fish fed the VOCH+ diet suggests that the combination of both VO and CH in the diet may negatively affect intestinal function. This seems of relevance in the context of alternative aquafeeds for gilthead sea bream, which tend to include increasing amounts of plant ingredients. Therefore, in future studies, it is important to clarify the interaction between CH and VO, and of which VO constituents are responsible for such effects. The long-term implications of such changes on fish digestive function and health should also be assessed.

Funding information This work was co-financed by the European Regional Development Fund (ERDF) through the COMPETE—Operational Competitiveness Programme and national funds through FCT—under the project "PEst-C/MAR/LA0015/2011". C.C., A.C., and CRS were supported by grants (SFRH/BPD/114942/2016, SFRH/BPD/101354/2014, SFRH/BPD/101038/2014 respectively) from FCT. A.D. was supported by the National Counsel of Technological and Scientific Development (CNPq), Brazil.

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