Fish and Shellfish Immunology 89 (2019) 187–197

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of dietary lipid sources on the intestinal microbiome and health of golden pompano (*Trachinotus ovatus*)

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ARTICLE INFO

Keywords:
Trachinotus ovatus
Dietary lipid source
Intestinal health
Intestinal microbiota
Inflammation

ABSTRACT

Replacement of fish oil (FO) with vegetable oils (VO) in diets is economically desirable for the sustainable development of the aquaculture industry. However, inflammation provoked by FO replacement limited its widely application in fish industry. In order to understand the mechanism of VO-induced inflammation, this study investigated the impact of different dietary vegetable oils on the intestinal health and microbiome in carnivorous marine fish golden pompano (*Trachinotus ovatus*). Three diets supplemented with fish oil (FO, rich in long-chain polyunsaturated fatty acids), soybean oil (SO, rich in 18:2n-6) and linseed oil (LO, rich in 18:3n-3), respectively, were fed on juvenile golden pompano for 8 weeks, and the intestinal histology, digestive enzymes activities, immunity and antioxidant indices as well as intestinal microbiome were determined. The results showed that dietary SO significantly impaired intestinal health, and decreased the number and height of intestinal folds, and muscle thickness, as well as the zonula occludens-1 (*zo-1*) mRNA expression in intestine. Moreover, the two dietary VO significantly decreased the amylase and lipase activities in intestine, and reduced the trypsin activity in the dietary SO group. Furthermore, the two VO diets increased intestinal acid phosphatase (ACP) activity, while intestinal lysozyme (LZM) activity and serum diamine oxidase (DAO) activity in the SO group were also significantly increased ($P < 0.05$). Analysis of the intestinal microbiota showed that the two VO diets significantly increased the abundance of intestinal potentially pathogenic bacteria (*Mycoplasma* and *Vibrio*) and decreased proportions of intestinal probiotics (*Bacillus and Lactococcus*), especially in the dietary SO group. These results indicate that complete replacement of FO with VO in diets would induce intestinal inflammation and impair intestinal function, which might be due to changes in intestinal microbiota profiles, and that dietary SO would have a more negative effect compared to dietary LO on intestinal health in *T. ovatus*.

1. Introduction

Dietary lipids are principal sources of energy, which also provides essential fatty acids for growth and development in fish [1]. Fish oil is the traditional dietary lipid source commonly used in the aquafeed industry to promote “protein sparing effects” [2]. However, inadequacy in the supply of fish oil (FO) and fish meat for aquatic production is viewed as a major obstacle to sustainable development of farmed fish, especially, the insufficient global supply of FO required to meet the high demands of the growing aquaculture industry [3,4]. There is therefore great interest in finding alternative sustainable sources of dietary lipids that could appropriately replace dietary FO for farmed fish. Vegetable oils from soybean, linseed, corn, sunflower and perilla are extensively used in manufacturing of aquaculture diets. Unlike FO, these vegetable oils are rich in polyunsaturated fatty acids with 18 carbon atoms (C18 PUFA), namely linolenic acid (LNA; 18:3n-3) and linoleic acid (LA; 18:2n-6), but lack long-chain polyunsaturated fatty acids (LC-PUFA) [5]. So far, vegetable oils (VO) are considered as the ideal alternative of FO in aquafeeds, due to their availability and relatively low price.

It has been demonstrated that soybean oil (SO) and linseed oil (LO) may be used to substitute for FO at different levels in diets, with

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https://doi.org/10.1016/j.fsi.2019.03.060
Received 28 December 2018; Received in revised form 19 March 2019; Accepted 26 March 2019

negligible negative effects on fish growth and requirements for essential fatty acid (EFA), especially in some freshwater and salmonid species [6–8]. But fish oil sparing by VO in diets has also been shown to be difficult in some fish species, which would have a negative impact on fish growth and meat quality, especially in marine carnivorous fish like white seabass *Atractoscion nobilis* [4,9]. The question is, how does dietary VO have an influence on the growth and lipid metabolism in fish, as so far, little is known about the effects of dietary VO on gut microbial community, intestine health and lipid absorption in fish [10].

Gut microbiota is known to play an important role in promoting host health and well-being. The intestinal microbiota mainly impacts on the host in terms of providing essential nutrient and non-nutrient factors, as well as increasing the host's ability to harvest nutrients from diets, by producing digestive enzymes, altering gut histology and forming a physical barrier to provide a defense against pathogens [11,12]. Historically, diets are believed to have a strong influence on the community structure of gut microorganisms, with the gut microbiome partly determined by dietary preferences [13–15]. Thus, the gut microbiome is an integral component of the host, and has received increasing attentions due to its importance in maintaining intestinal homeostasis [16–18]. The intestinal homeostasis is believed to be beneficial to the immune system and for optimal nutrient uptake, which are necessary for normal growth and health of the host [19,20]. Disturbance of the intestinal homeostasis may change the intestinal physiology including the intestinal microbiome, mucosal barrier and local immune system of the host, thereby causing a disorder in the metabolism of nutrients and disease resistance [21,22].

Previous studies have shown that the levels of dietary lipids could affect the composition of intestinal microbiota in aquaculture animals. Sun et al. (2018) reported that, increasing of dietary lipid levels resulted in a decrease in the abundance of Fusobacteria, and an increase in the levels of Proteobacteria, as well as an elevation in the abundance of pathogenic bacteria in intestine, thereby increasing the potential risk of disease in swimming crab *Portunus trituberculatus* [23]. Similarly, it has been shown that when Arctic charr *Salvelinus alpinus* L. fed diets with different lipid sources such as soybean oil, linseed oil and fish oil, different microbiota strains become dominant in the gut, with *carnobacteria* isolated only from the hindgut region of fish fed on soybean oil and linseed oil, and with variations in bacterial colonization of the enterocyte surface in the different groups [24]. These results suggest that dietary lipids might have an impact on gut microbiota and therefore on intestinal health of fish and other aquaculture animals.

Golden pompano (*Trachinotus ovatus*), a widely cultured carnivorous marine fish along the south-eastern coast of China, has become an economically important fish species in the aquaculture industry due to its fast growth, high flesh quality, wide salinity tolerance and suitability for cage culture [25,26]. In the present study, three diets supplemented with fish oil (FO, rich in long-chain polyunsaturated fatty acids), soybean oil (SO, rich in 18:2n-6) and linseed oil (LO, rich in 18:3n-3), were fed to juvenile golden pompano for 8 weeks, and the intestinal microbiome, histological structure, digestive enzymes activities, immunity and antioxidant indices were determined, to investigate the effects of these dietary lipid sources on the intestinal health and microbial community in golden pompano. The aim was to investigate how intestinal microbiota responds to different dietary lipid sources in carnivorous marine fish, and then to affect the intestinal health. The results of this study will provide novel insights into the nutritive properties of vegetable oils, and offer indications on how to implement more efficient FO replacement strategies in aquaculture feeds.

2. Materials and methods

2.1. Experimental diets and feeding trial

Three isonitrogenous and isolipidic diets were formulated to contain different lipid sources, fish oil (Named VF group, as the control group), soybean oil (Named VS group) and linseed oil (Named VL group), respectively (Table 1). Diets were prepared and stored following the standard procedures of our laboratory, as previously reported [27]. Fatty acid composition of diets was analyzed following the method described by Xu et al. (2012) and shown in Table 2 [7].

Table 1
Ingredients and proximate composition of the experimental diets (% dry matter).

Ingredients (%)	VF	VS	VL
Casein	41.00	41.00	41.00
Fermented soybean meal	21.00	21.00	21.00
Cassava starch	11.00	11.00	11.00
α-Starch	3.00	3.00	3.00
Fish oil[a]	9.00	0	0
Soybean oil[b]	0	9.00	0
Linseed oil[c]	0	0	9.00
Soybean lecithin	2.00	2.00	2.00
Choline chloride	0.50	0.50	0.50
Betaine	0.50	0.50	0.50
$Ca(H_2PO_3)_2$	1.00	1.00	1.00
Lutein	0.20	0.20	0.20
Vitamin premix[d]	2.00	2.00	2.00
Mineral premix[d]	2.00	2.00	2.00
Microcrystalline cellulose	6.80	6.80	6.80
Proximate compositions (dry matter basis)			
Crude protein	50.04	50.82	50.73
Crude lipid	11.55	12.21	12.35
Ash	4.56	4.72	5.04

[a] Fish oil: 28.8% saturated fatty acids, 37.7% monounsaturated fatty acids, 0.8% C18:3n-3, 1.2% C18:2n-6, 17% C20:5n-3, 8.8% C22:6n-3 and other fatty acids.

[b] Soybean oil: 60.79% C18:2n-6, 7.67% C18:3n-3 and other fatty acids.

[c] Linseed oil: 15.85% C18:2n-6, 55.52% C18:3n-3 and other fatty acids.

[d] The vitamin and mineral premixes were obtained from Yuequn Ocean Biological Research Development Co., Ltd., Jieyang, Guangdong, China.

Golden pompano juveniles were obtained from a sea-floating farm in Zhangzhou, Fujian, China. Prior to the start of the normal feeding experiment, fish were acclimated to an equal mixture of the three experimental diets for two weeks. Next, the fish were fasted for 24 h and individually weighed after anesthesia in 0.01% 2–phenoxyethanol (Sigma–Aldrich, USA). A total of 225 fish (initial weight 8.80 ± 0.50 g) were randomly distributed to 9 net cages (1.0 m × 1.0 m × 1.5 m) with 25 individuals per cage. Each experimental diet was randomly assigned to three net cages. The feeding trial lasted for 8 weeks. During the feeding period, fish were slowly hand-fed to apparent satiation twice daily (6:30 and 18:00).

2.2. Sample collection

At the end of the feeding trial, 6 h after the last feeding, two fish per cage (six fish per treatment) were randomly selected and collected for analysis of intestinal microbiota. The fish were anesthetized with 0.01% 2–phenoxyethanol, and then separately weighed. For the analysis of intestinal microbiota, the surface of the fish was sterilized by tampon with 70% alcohol, then the abdominal cavity of fish was opened, and the whole intestine was removed by using sterile scissors and bistoury. The whole intestinal content of fish from foregut region to hindgut region was carefully squeezed out using sterile tweezers, and then transferred to 2 ml sterile tubes (Axygen, America). Next, the intestine was flushed with sterile phosphate-buffered saline with a 1 ml syringe to remove all remnants of intestinal content into the sterile tubes.

In addition, after the last feeding, the residual fish from each cage were starved for 24 h. The fish were anesthetized with 0.01% 2–phenoxyethanol, and then euthanized by a sharp blow to the head before being sampled. Two fish per cage (six fish per treatment) were randomly selected to collect serum. Bloods was withdrawn from the caudal

Table 2
Fatty acid composition of the experimental diets (%).

	VF	VS	VL
C6:0	0.07 ± 0.01	0.06 ± 0.02	0.06 ± 0.00
C10:0	0.20 ± 0.01	0.19 ± 0.01	0.19 ± 0.01
C12:0	0.22 ± 0.00^{a}	0.11 ± 0.00^{b}	0.12 ± 0.00^{b}
C13:0	0.53 ± 0.13	0.32 ± 0.07	0.40 ± 0.12
C14:0	5.39 ± 0.06^{a}	0.67 ± 0.00^{b}	0.66 ± 0.03^{b}
C15:0	0.64 ± 0.00^{a}	0.11 ± 0.01^{b}	0.12 ± 0.00^{b}
C16:0	21.15 ± 0.15^{a}	12.31 ± 0.11^{b}	8.50 ± 0.03^{c}
C18:0	5.25 ± 0.09	4.97 ± 0.09	4.57 ± 0.03
C22:0	1.49 ± 0.04	ND	ND
C23:0	0.79 ± 0.01	ND	ND
ΣSFA[a]	35.75 ± 0.34^{a}	19.24 ± 0.19^{b}	15.07 ± 0.19^{c}
C14:1	0.24 ± 0.01^{a}	0.11 ± 0.00^{b}	0.07 ± 0.00^{c}
C16:1	4.80 ± 0.10^{a}	0.24 ± 0.01^{b}	0.21 ± 0.00^{b}
C18:1	18.83 ± 0.11^{a}	20.50 ± 0.01^{b}	17.47 ± 0.05^{a}
C20:1	1.80 ± 0.02^{a}	0.49 ± 0.01^{b}	0.19 ± 0.00^{c}
C24:1n-9	0.34 ± 0.02	ND	ND
ΣMUFA[b]	26.17 ± 0.21^{a}	21.34 ± 0.02^{b}	17.93 ± 0.06^{c}
C18:3n-3	6.60 ± 0.03^{a}	6.99 ± 0.06^{a}	45.35 ± 0.20^{b}
C20:3n-3	0.22 ± 0.01^{a}	0.32 ± 0.02^{a}	0.13 ± 0.00^{b}
C20:5n-3	7.64 ± 0.09^{a}	0.14 ± 0.01^{b}	0.09 ± 0.00^{b}
C22:6n-3	8.89 ± 0.04	ND	ND
Σn-3PUFA[c]	23.34 ± 0.12^{a}	7.45 ± 0.03^{b}	45.57 ± 0.20^{c}
C18:2n-6	11.98 ± 0.43^{a}	50.55 ± 0.13^{b}	20.63 ± 0.04^{c}
C20:3n-6	0.25 ± 0.02	ND	ND
C20:4n-6	2.23 ± 0.05	ND	ND
Σn-6PUFA[d]	14.60 ± 0.41^{a}	50.55 ± 0.13^{b}	20.63 ± 0.04^{c}
ΣPUFA[e]	37.95 ± 0.53^{a}	58.00 ± 0.17^{b}	66.20 ± 0.24^{c}

Note: All values are means ± SD (n = 4). ND, no detected. Means in the same line with different superscript letters are significantly different ($P < 0.05$) as determined by Duncan's test.

[a] ΣSFA is the sum of saturated fatty acids.

[b] ΣMUFA is the sum of monounsaturated fatty acids.

[c] Σn-3PUFA is the sum of n-3 polyunsaturated fatty acids.

[d] Σn-6PUFA is the sum of n-6 polyunsaturated fatty acids.

[e] ΣPUFA is the sum of polyunsaturated fatty acids.

vein of fish and stored at 4 °C for 4 h. Serum was collected after centrifugation (4000 *g*, 10 min) and stored at 4 °C until use. The middle part (about 1 cm in length) of anterior intestine from 4-four blood-withdrawn fish per treatment were dissected and fixed in 4% paraformaldehyde solution for histological evaluation. The anterior intestine of four other fish per treatment were collected for analysis of the digestive, immune and antioxidative-related indices, especially enzymes activities. To analyze gene expression in intestine, the whole gut from six fish per treatment were collected following the procedure described above. The samples for determining enzymatic activity and gene expression, were immediately frozen in liquid nitrogen and stored at −80 °C until use, while the serum samples for the diamine oxidase (DAO) analysis were stored at 4 °C.

2.3. Intestinal histology

The anterior intestine samples were fixed in 4% paraformaldehyde solution for 48 h, and according to the histological technique, samples were then dehydrated in ethanol, equilibrated in xylene and embedded in paraffin. Sections with 5 μm thickness of anterior intestine were cut and stained with hematoxylin and eosin (H & E). The slides were then examined and photographed under a light microscope (Axio Imager 2, Zeiss, Oberkochen, Germany) equipped with camera (Axiocam 506, Zeiss, Oberkochen, Germany) and image acquisition software (ZEN 2, Zeiss, Oberkochen, Germany) for the presence of degenerative changes in epithelial cells or intestinal physiology according to previously reported criteria [28,29]. The following features were evaluated: (1) damaging and shortening of the intestinal folds; (2) loss of the supranuclear vacuolization in absorptive cells (enterocytes) of the intestinal epithelium; and (3) widening of intestinal wall thickness. The height and number of intestinal folds were measured from the whole section of anterior intestine, and goblet cell number were measured from ten random intestinal folds in each section of anterior intestine (ten section each fish, four fish in each group). In addition, thicknesses of intestinal wall in ten micrographs for each fish were measured. For each diet, forty sections originating from four fish (n = 40) were analyzed.

2.4. Assay of immune-related enzyme, digestive enzyme activities and antioxidant indices in intestine and serum

Serum samples were assayed within 24 h after collection and storage at 4 °C. The DAO activity of serum was determined using assay kits (Nanjing Jiancheng Bioengineering Institute, China). Intestinal samples were homogenized in ice-cold physiological saline 0.89% (w/v) buffer, and the homogenate was centrifuged for 20 min at 3000 r/min to collect the supernatant. The supernatant was then used to quantify intestinal digestive enzyme activities (amylase, lipase and trypsin), immune-related enzyme activities (lysozyme, acid phosphatase and alkaline phosphatase) and the parameters of antioxidative ability (malondialdehyde, total-superoxide dismutase, glutathioneperoxidase, glutathione) using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China). All the enzymatic activities and non-enzymatic factor were calculated according to the manufacturer's instructions.

2.5. RNA isolation and gene expression analysis

Total RNA from intestine was isolated with a BioFast Simply P Total RNA Extraction kit (BioFlux, Hangzhou, China) according to the manufacturer's instructions. The RNA quality was assessed by formaldehyde agarose gel electrophoresis, and the concentration of RNA was quantified with a spectrophotometer (NanoDrop 2000, Thermo Fisher, Germany). First-strand cDNA was synthesized from 1 μg total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) following the manufacturer's instructions. The obtained cDNA templates were then stored at −80 °C for later use. Quantitative real-time PCR (*q*PCR) was carried out on the LightCycler® 480 thermocycler (Roche, Germany) in a total volume of 10 μL with the LightCycler® 480 SYBR Green I Master (Roche, Germany) following the manufacturer's protocol. All amplification reactions were run in triplicate. Before the *q*PCR amplification, the specificity and efficiency of the primers for the *β-actin* and target genes were detected by constructing a standard curve using serial dilution of cDNA, and the standard equation and correlation coefficient for each primer pair were determined. The specific primers of the *β-actin* gene and target genes are listed in Table 3. The relative mRNA expression of target genes was normalized to the *β-actin* mRNA, and determined using the $2^{-\Delta\Delta CT}$ method.

2.6. Bacterial DNA extraction, PCR amplification, and sequencing

Total bacterial community DNA was extracted from the whole intestinal content of each fish with a TIANamp Micro DNA Purification Kit (Tiangen, Beijing, China). The DNA concentration and purity were detected on 1% agarose gels. The total DNA was diluted to 1 ng/μL, and then was sent for high-throughput sequencing at Novogene Biological Information Technology Co. (Beijing, China).

Bacterial DNA was used as template for amplification of the V4 region of the 16S rRNA gene with the primers 515F (5’ - GTGCCAGC MGCCGCGGTAA - 3′) and 806R (5’ - GGACTACHVGGGTWTCTAAT - 3′). All PCR reactions were carried out in 30 μL volume with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, approximately 10 ng template DNA, and nuclease-free water. Thermal cycling conditions were as follows: denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and next extension at 72 °C for 60 s, then a final extension at 72 °C for 5 min. The quantity and

Table 3
Primers used for quantitative real-time PCR (*q*PCR).

Target genes	Forward primer (5′–3′)	Reverse primer (5′–3′)	references
zo-1	CGACAAAGAGAAAGGAGAAACG	AGAAGTGGTCAATGAGCACAGATA	[26]
ocln	TACGCCTACAAGACCCGCA	CACCGCTCTCTCTGATAAA	[26]
cdh1	CCCTGCTGGTGTTTGACTAT	GCAGTCGTAATCCTGGTCTC	[a]
il8	TGCATCACCACGGTGAAAAA	GCATCAGGGTCCAGACAAATC	[26]
tnf α	GCTCCTCACCCACACCATCA	CCAAAGTAGACCTGCCCAGACT	[26]
c4	TGGAGAAAAAGTTAAAGGGGC	CAGGAAGGAAGTATGAGCGAGT	[26]
lyz	GGAGTCTGGTGTTTCTGCTCTTTG	GGTGGCTCTAGTGTTGTAGTTCG	[26]
fabp2	CGGCTCCTGGAAAATTGATC	ATGGTTATCTTGAGGTTGTCGTG	MF034871
pparα	AATCTCAGCGTGTCGTCTT	GGAAATGCTTCGGATACTTG	KP893147
alp	AGCAGGTAAATCAGTGGGAATAG	GGGAGATCAGCGTCAGAATAC	[a]
B-actin	CGCCCGAGTGTGTATGAGAAATG	GTCATGTGGATCAGCAAGCAGGA	KX987228

[a] Supplementary data.

purity of the PCR product were assessed using a Gene JET Gel Extraction Kit (Thermo Scientific, Carlsbad, CA, USA). Sequencing libraries were generated with the Illumina TruSeq® DNA PCR-Free Library Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index codes were added. The library quantity was assessed on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and quality on an Agilent Bioanalyzer 2100 system. The library was then sequenced on an Illumina HiSeq 2500 PE250 platform at Beijing Novogene Genomics Technology Co. Ltd. (China).

2.7. Microbial community composition analysis

In general, analysis of the sequence data was performed as described elsewhere [30]. Briefly, sequencing reads were assigned to samples based on the unique barcode of each sample and truncated by cutting off the barcode and primer sequence. Merged reads from the original DNA fragments were used as raw tags with FLASH V.1.2.7 (http://ccb.jhu.edu/software/FLASH/). Quality filtering was first analyzed according to QIIME V1.7.0 (http://qiime.org/index.html), and then quality filtering on the raw tags were executed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME V1.7.0 quality controlled process [31]. Chimera sequences were detected and removed using an UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to obtain the high-quality effective tags [32]. The tags were classified into the same OTUs (operational taxonomic units) using UPARSE V7.0.1001 (http://drive5.com/uparse/) based on an identity threshold of 97% similarity [31]. The GreenGene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) was used to screen each representative sequence based on a RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) algorithm for further annotation. The analyses including clustering, alpha diversity (Chao1 index, observed species number, Shannon index, Simpson and ACE) and beta diversity (PCoA) were calculated with QIIME (v1.7.0) and displayed with R software (v2.15.3). Rarefaction curves were generated based on observed species. Statistical differences of α diversity and β diversity between treatments were performed according to Tukey's test and Wilcox's test. Beta diversity between bacterial communities was identified using weighted Unifrac distances. Hierarchical clustering of samples was performed using UPGMA (unweighted pair-group method with arithmetic means). PCoA on the Unifrac distances of the weighted distance matrices was evaluated to visualize differences in bacterial community composition and structure. In addition, to assess the changes in microbial community structure brought by dietary VO, differentially abundant taxa between the control group and the VO groups were evaluated using T-test analysis and Metastats analysis [33,34].

2.8. Statistical analysis

Data are presented as mean ± SEM (standard error), and analyzed using SPSS 17.0 software. All statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey and Duncan's tests. Correlations of separate dietary fatty acid levels and intestinal microbiota communities were analyzed by Pearson correlation analysis. The statistical significance level was set at $P < 0.05$.

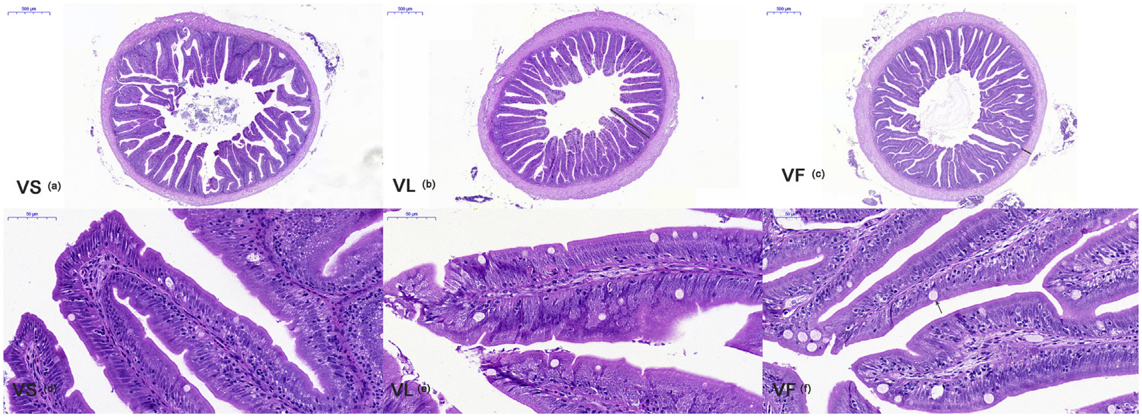

Fig. 1. The anterior gut morphology of *Trachinotus ovatus* fed diets with different lipid sources: VS, soybean oil; VL, linseed oil; VF, fish oil. (a), (b) and (c) is whole anterior histological section of VS, VL and VF group under 5× magnification, respectively, and the below picture (d), (e) and (f) is part of (a), (b) and (c) under 40× magnification, respectively. The locations of the black line and arrow in pictures (b), (c) and (f) point to the intestinal fold height, wall thickness and goblet cell, respectively.

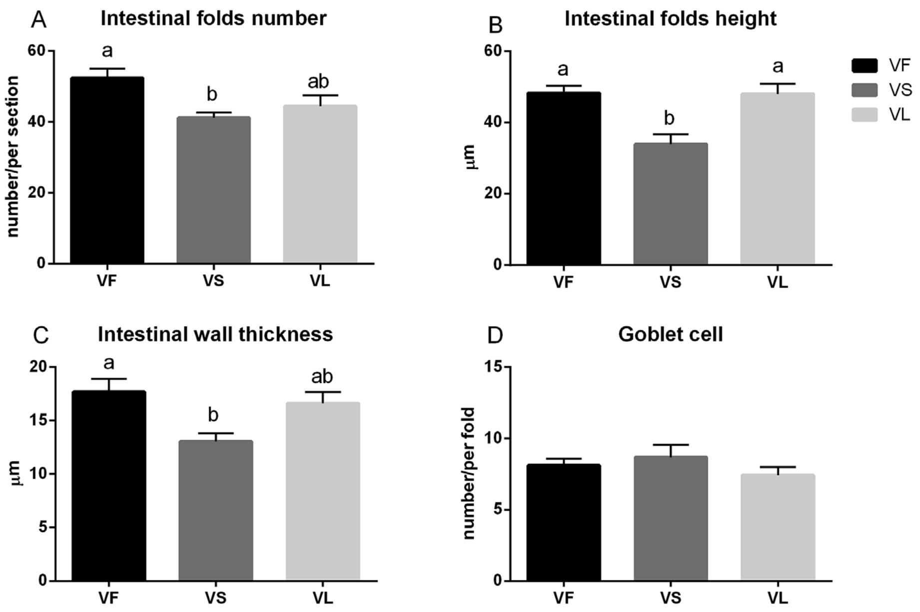

Fig. 2. Anterior gut morphology parameters of *Trachinotus ovatus* fed diets with different lipid sources for 8 weeks (mean ± S.E. of three replications). Columns with different letters are significantly different ($P < 0.05$).

3. Results

3.1. Effects of dietary VO relative to dietary FO on intestinal histology of golden pompano

The results of intestinal histology are shown in Figs. 1 and 2. It can be seen that the histology of anterior intestine showed a high degree of damage in response to dietary soybean oil (VS group), but not to dietary linseed oil (VL group), when compared to fish fed diets with fish oil (VF group). The height and number of intestinal folds and intestinal wall thickness in the anterior intestine were significantly decreased in the VS group ($P < 0.05$), while no significant difference was observed between the VL and VF groups ($P > 0.05$). Furthermore, there were no significant difference in the goblet cells in every fold among the VS, VL and VF groups ($P > 0.05$).

3.2. Effects of dietary VO on serum and intestinal immune-related enzyme, intestinal digestive enzyme activities and antioxidant indices of golden pompano

In general, significant differences were observed in the digestive enzyme activities between the fish oil and vegetable oil groups (Fig. 3). For example, the VS and VL groups showed much lower amylase and lipase activities than the VF group ($P < 0.05$). The lowest trypsin activity was also observed in the VS group, which was 27.3% lower compared to the VF group.

The two dietary VO elevated the intestinal acid phosphatase (ACP) activity, as well as the activities of the intestinal lysozyme (LZM) and serum diamine oxidase (DAO) in the VS group ($P < 0.05$). On the other hand, the alkaline phosphatase (ALP) activity decreased significantly ($P < 0.05$) in the VL group (Fig. 4). However, no significant difference ($P > 0.05$) was detected in the parameters of antioxidative ability (MDA, T-SOD, GSH, GSH-PX) in intestine among the three groups (Fig. 5).

3.3. Effects of dietary VO on the mRNA expression of genes related to lipid nutrient uptake, immunity and tight junction in intestine of golden pompano

Intestinal fatty acid-binding protein (*fabp2*) and peroxisome proliferator activated receptors-alpha (*pparα*) are lipid absorption-related genes (Fig. 6A). There was no significant difference in the mRNA expression of *pparα* and *fabp2* between the two dietary VO groups and the VF group. However, the *fabp2* mRNA levels in intestine of the VL group was significantly lower than that of the VS group ($P < 0.05$). Moreover, the mRNA level of intestinal-type alkaline phosphatase (*alp*) in intestine was significantly ($P < 0.05$) up-regulated in the VS group, when compared with the VL and VF groups, respectively (Fig. 6A).

Tumor necrosis factor α (*tnfα*), interleukin 8 (*il-8*), complement 4 (*c4*) and lysozyme (*lyz*) are immunological or inflammatory status-related genes. There was no significant difference ($P > 0.05$) in the transcript levels of *tnfα*, *il*-8, *c4* and *lyz* in intestine of the three groups (Fig. 6B).

The mRNA levels of intestinal epithelial barrier related genes in intestine of golden pompano are shown in Fig. 6C. The mRNA levels of

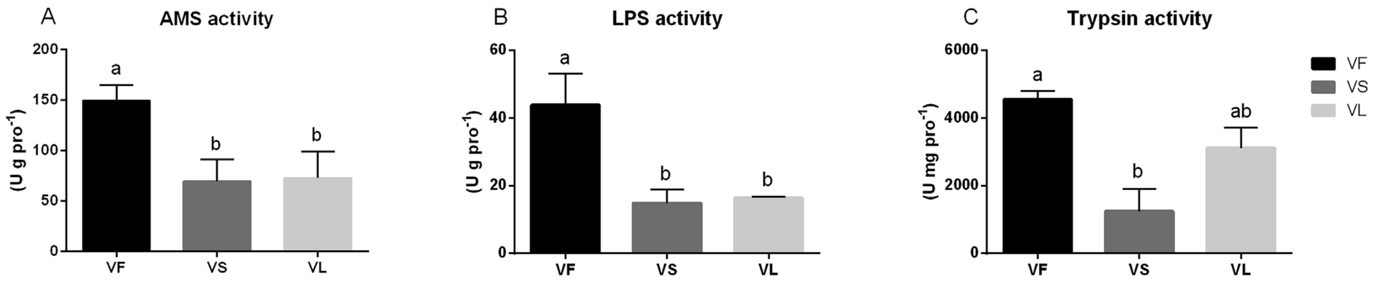

Fig. 3. Intestinal digestive enzymes activities of *Trachinotus ovatus* fed diets with different lipid sources for 8 weeks (mean ± S.E. of three replications). Columns with different letters are significantly different ($P < 0.05$). AMS, amylase; LPS, lipase.

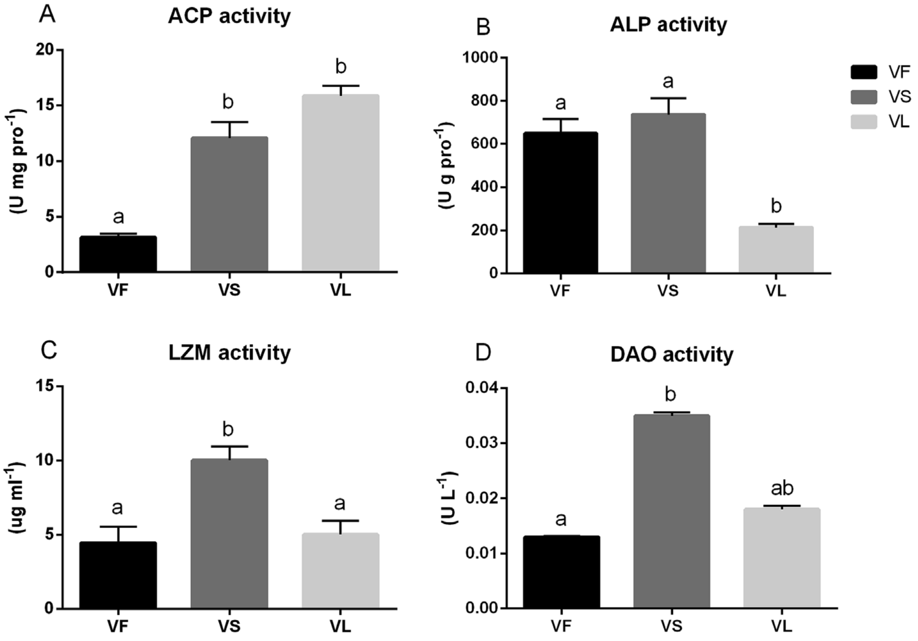

Fig. 4. Immune-related enzymes activities in intestine or serum of *Trachinotus ovatus* fed diets with different lipid sources for 8 weeks (mean ± S.E. of three replications). Columns with different letters are significantly different ($P < 0.05$). ACP, acid phosphatase; ALP, alkaline phosphatase; LZM, lysozyme; DAO, diamine oxidase.

zonula occludens-1 (*zo-1*) were significantly down-regulated in the VS group ($P < 0.05$), while the mRNA levels of occludin (*ocln*) and cadherin 1 (*cdh1*) showed no significant difference among the three groups ($P > 0.05$).

3.4. Intestinal microbial community structures of golden pompano fed the three experimental diets

In general, abundant microbial groups were detected in the gut of golden pompano fed the three diets, with the highest estimated richness from the VL group (as determined by the ACE index; Table 4; $P < 0.05$). However, bacterial diversity of the VS group decreased significantly as compared to the VF and VL groups (as determined by Shannon diversity index; $P < 0.05$). On phyla level, Proteobacteria, Tenericutes and Firmicutes were detected as the dominant phyla in intestine of the golden pompano (Fig. 7A). Firmicutes were the most dominant in the VF group, while Tenericutes were the most dominant both in the VL and VS groups. The proportion of Firmicutes ranged from 72.35% to 27.89%, 12.55%, followed by Tenericutes with the proportion ranging from 8.62% to 47.86%, 63.86% in the VF, VL and VS groups, respectively. On genus level, the predominant genera of golden pompano intestinal microbiota communities composed of *Mycoplasma*, *Photobacterium*, *Bacillus*, *Streptococcus*, *Vibrio*, *Brevinema*, *Lactococcus*, *Lactobacillus* and *Globicatella* (Fig. 7Band C). *Mycoplasma* and *Bacillus* were detected as the most dominant genera, with the proportion of *Mycoplasma* ranging from 8.59% to 47.81%, 63.86%, followed by *Bacillus* with the proportion ranging from 17.82% to 3.85%, 2.27% in the VF, VL and VS groups, respectively.

To explore the similarity in the microbial community composition of 15 specimens, the PCoA was performed using Weighted UniFrac distances. The PCoA cluster (Fig. 8) analysis revealed that samples clustered together according to the diets with a clear separation between them, and with the microbiota community of VS group more similar to the VL group than to the VF group. Dietary soybean oil and

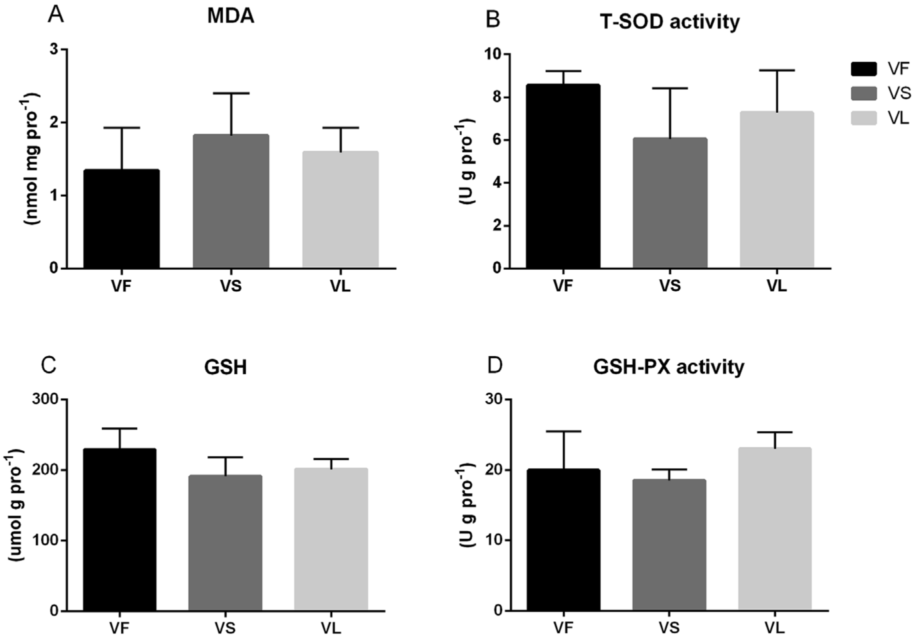

Fig. 5. The parameters of intestinal antioxidative ability of *Trachinotus ovatus* fed diets with different lipid sources for 8 weeks (mean ± S.E. of three replications). Columns with different letters are significantly different ($P < 0.05$). MDA, malondialdehyde; T-SOD, total superoxide dismutase; GSH, glutathione; GSH-PX, glutathion peroxidase.

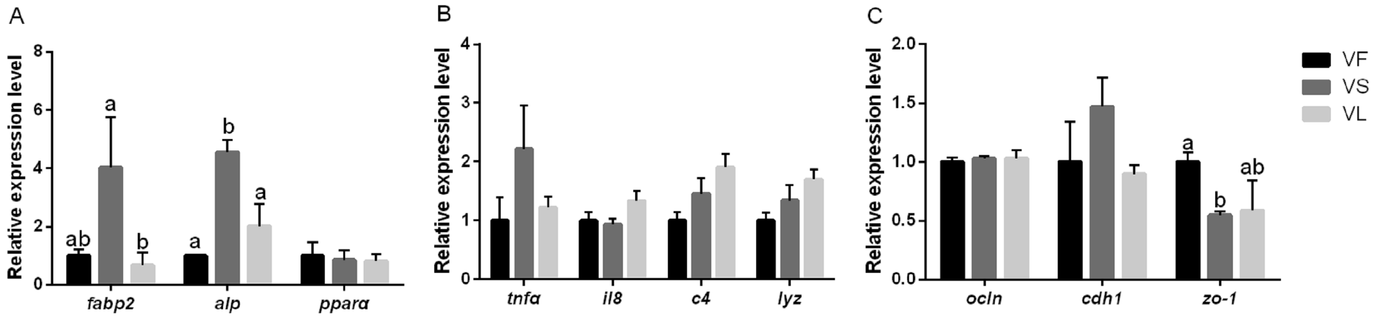

Fig. 6. Relative mRNA expression of *fabp2*, *alp*, *pparα* (a) and *tnfα*, *il-8*, *c4*, *lyz* (b) and *zo-1*, *ocln*, *cdh1* (c) in intestine of *Trachinotus ovatus* fed diets with different lipid sources for 8 weeks. Values are means (n = 6), and bars with different letters within the same gene expression denote a significant difference among treatments ($P < 0.05$). VS: soybean oil; VL: linseed oil; VF: fish oil.

Table 4
Alpha diversity index of intestinal microbiota of *Trachinotus ovatus* fed different diets.

Sample name	Richness estimates			Diversity estimates
	OTUs	Chao1	ACE	Shannon
VF	383 ± 24	484 ± 34	457 ± 38^a	4.26 ± 0.19^a
VS	293 ± 30	424 ± 36	452 ± 26^a	2.77 ± 0.31^b
VL	454 ± 82	594 ± 79	676 ± 83^b	3.81 ± 0.49^{ab}

Note: data in the same column with different superscript letters are significantly different ($P < 0.05$).

linseed oil had different effects on the overall structure of intestinal microbiota in golden pompano, with the latter much closer to the effect of dietary fish oil.

Different groups showed significant differences on genus and species level in bacterial communities based on further MetaStat analysis. Dietary soybean oil significantly increased the relative genus abundance of *Mycoplasma*, *Vibrio* and *Brevinema* ($P < 0.05$), and decreased significantly the relative abundance of *Bacillus*, *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Globicatella* and *Leuconostoc* ($P < 0.05$), when compared with dietary fish oil (Fig. 9A). Furthermore, dietary linseed oil also significantly increased the relative genus abundance of *Mycoplasma* and *Vibrio* ($P < 0.05$), and decreased significantly the relative abundance of *Bacillus*, *Streptococcus*, *Lactococcus*, *Globicatella* and *Leuconostoc* ($P < 0.05$), when compared with dietary fish oil (Fig. 9C). Meanwhile, significant decline in abundance of species *Bacillus_velezensis*, *Streptococcus_salivarius*, *Lactococcus_raffinolactis*, *Lactococcus_lactis* and *Leuconostoc_mesenteroides* were also observed in the VS and VL groups compared with the VF group (Fig. 9B and D). MetaStat analysis revealed that dietary soybean oil and linseed oil had no significant effects on taxonomic distribution of intestinal microbiota communities.

Correlation analysis of the individual dietary fatty acid content and intestinal predominant microbial genera in golden pompano showed that dietary C20:4n-6, C20:5n-3 and C22:6n-3 contents have a significantly positive correlation with the genus *Leuconostoc*, *Globicatella*, *Lactococcus* and *Bacillus*, while a significantly negative correlation was observed with the genera *Mycoplasma* (Table 5).

4. Discussion

4.1. Dietary VO affects intestinal histology, and reduces digestion efficiency and absorption of nutrients

The integrity of the intestinal histology plays a crucial role in maintaining normal functions of the gut [35]. Damage to the intestinal histology could cause shorter villi and deeper crypts due to the presence of toxins [36]. An increase in intestinal muscle thickness, and number and height of intestinal folds are an indication of increasing surface area for nutrient absorption, and then improving intestinal digestion and absorption physiology [35–37]. In the present study, the height and number of intestinal folds as well as intestinal wall thickness in the foregut were significantly decreased in the VS (dietary soybean oil) group. However, there was no significant difference observed between the VL (dietary linseed oil) and VF (dietary fish oil) groups (Fig. 2). Moreover, the two dietary VO groups exhibited lower amylase and lipase activities, as well as lower trypsin activity in the VS group, compared with the VF group. The results suggest that dietary supplementation with VO might play a negative role in intestinal digestion and the absorption ability of golden pompano, especially in the dietary soybean oil group. PPAR serves as a monitor of intracellular non-esterified fatty acids (NEFA) levels, as free fatty acids bind to PPAR resulting in changes of the transcription levels of many target genes involved in lipid metabolism and storage [38]. As a transporter, FABP2 might play a role in directing fatty acids entry into intestinal mucosa cells for metabolism [39]. In the present study, the mRNA expression of *pparα* and *fabp2* in the two dietary VO groups showed no significant difference with the VF group. The *pparα* and *fabp2* genes are not only involved in the transport metabolism of free fatty acids in intestine, but also involved in other aspects of lipid metabolism in animals, which is very complicated and remains to be explored.

Intestinal alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphomonoesters with the release of inorganic phosphate and

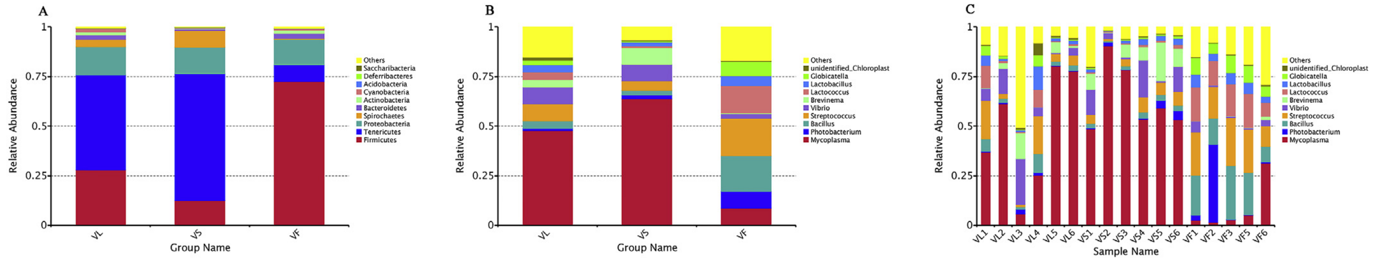

Fig. 7. Intestinal bacterial composition in three groups at the phylum (A) and genus (B&C) levels. Only the top 10 most abundant (Based on relative abundance) bacterial phyla and genera were shown. Other phyla and genera were all assigned as 'Others'.

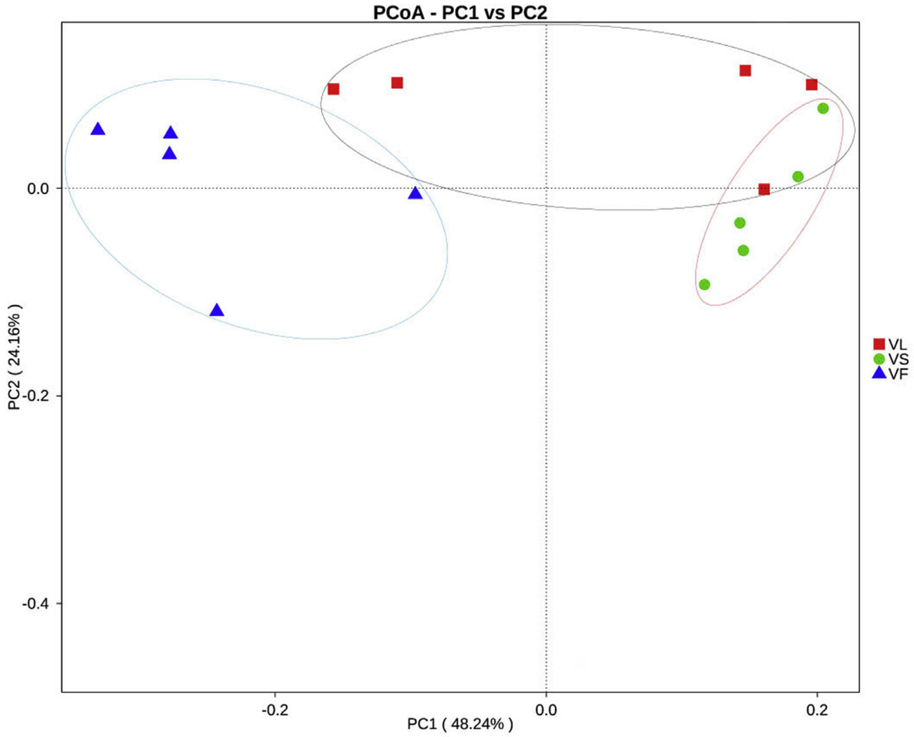

Fig. 8. Two-dimensional Principal co-ordinate analysis (PCoA), based on weighted-Unifrac distance, of the intestinal bacterial communities of *Trachinotus ovatus* fed diets with soybean oil (VS; green), linseed oil (VL; red) or fish oil (VF; blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

alcohol in intestine. Previous studies have reported that ALP could play a role in regulating the rate of lipid absorption [40,41]. For example, ALP-deficient mice could accelerate the transport of fat droplets through the intestinal epithelium and elevate serum triglyceride levels, and therefore gain faster body weight than wild-type mice [41]. Moreover, ALP also plays a crucial role in maintaining intestinal homeostasis to prevent bacterial invasion across the gut mucosal barrier [42,43]. In the present study, a decreased mRNA expression and enzymatic activity of ALP was observed in intestine of the VL (dietary linseed oil) group compared to the VS (dietary soybean oil) group. It thus seems dietary linseed oil might result in a faster rate of lipid absorption in golden pompano.

4.2. Dietary VO impairs intestinal immune function

As an immune organ, the intestine plays an important role in defense against pathogens [44]. In the present study, there was no significant difference in the mRNA expression of lysozyme (LZM) in intestine among the three groups (Fig. 6B). However, intestinal lysozyme activity was significantly elevated by dietary soybean oil (Fig. 4). Lysozyme acts as non-specific immune molecules against the incursion by detrimental bacteria, as it can attack the peptidoglycan of bacterial cell walls [45]. Many studies have reported that plant ingredients up-regulated the expression of pro-inflammatory cytokines and increased lysozyme activity [46,47]. Moreover, we also observed here, an elevation in the activities of immune-related enzyme ACP by the two dietary VO. Several researchers have reported that stronger ACP and ALP activities could enable phagocytes to destroy and more effectively clear pathogens, therefore conferring an increased resistance of fish to long-term pathogen invasion [48,49]. In the present study, the higher ACP and lysozyme activities in the VS group, indicates a strong intestinal inflammation response to dietary soybean oil in golden pompano, which necessitates stronger intestinal lysozyme and ACP activities relative to the FO group to prevent inflammation. In the present study, the mRNA expression of other immunity or inflammation related genes such as tumor necrosis factor α (*tnfα*), interleukin 8 (*il-8*) and complement 4 (*c4*) in intestine showed no significant difference among the three groups.

The most apical components of the junctional complex between adjacent epithelial cells that strengthen the epithelial mechanical barrier are tight junction (TJ) proteins (such as occludin, claudins and ZO-1), which provide one form of cell-cell adhesion in enterocytes and play a critical role in regulating paracellular barrier permeability [26,50]. Many studies have reported that decreased Occludin and ZO-1 expression and up-regulation in Claudin-1 mRNA levels impaired TJ barrier function in fish [26,51]. Similar results were observed in this study, as shown by a decline in the mRNA level of ZO-1 in the VS group (Fig. 6C). Moreover, the changes in the plasma diamine oxidase (DAO) level are associated with the functional injury of intestinal mucosal barrier and intestinal probiotics [52]. In the present study, the DAO activity level in serum was significantly increased in the VS group, indicating that dietary soybean oil was not beneficial for mechanical barrier physiology and intestinal health, as it could induce inflammation in golden pompano.

4.3. Dietary VO has a negative effect on intestinal microbiota by increasing the number of pathogens

The effects of three dietary lipid sources on intestinal bacteria community or diversity in golden pompano was explored in the present study using Illumina-based high-throughput sequencing. Compared with dietary FO treatment, more OTU numbers were observed in the gut of golden pompano fed diets containing linseed oil, with few OTU numbers found in the fish fed diets containing soybean oil (Table 4). This observation is consistent with previous reports, where linseed oil was shown to increase the diversity of intestinal microbiota of fish [53]. Meanwhile, the intestinal bacterial communities of golden pompano in the VS, VL and VF groups formed different clusters (Fig. 8). The VS and VL clusters were relatively coherent, while the VF cluster was clearly separated from the VS clusters, indicating that dietary soybean oil might have a more profound effect on the intestinal bacterial community of golden pompano relative to the effect of dietary linseed oil, when compared with dietary FO.

Furthermore, an abundance of the phyla Firmicutes, Proteobacteria

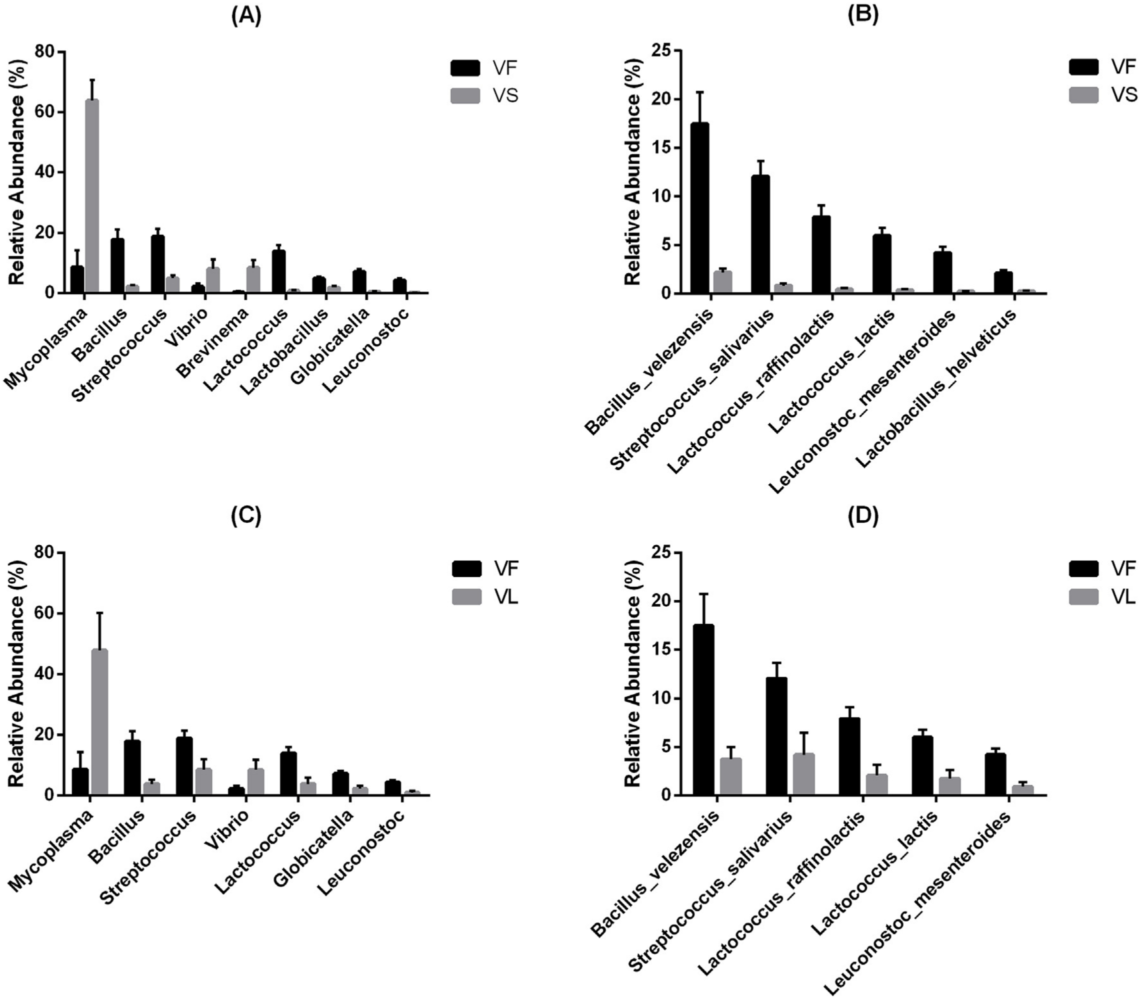

Fig. 9. MetaStat analysis of intestinal microbiota communities of *Trachinotus ovatus*. (A) and (C), the genus with significantly up or down-regulated abundance in group VS and VL, respectively, compared with group VF; (B) and (D), the species with significantly up or down-regulated abundance in group VS and VL, respectively, compared with group VF. Only the top abundant (based on relative abundance) bacterial genera and species were shown.

Table 5
Correlation analysis of individual dietary fatty acid content and predominant genera of intestinal microbiota communities of *Trachinotus ovatus*.

	Mycoplasma	*Bacillus*	*Streptococcus*	*Brevinema*	*Lactococcus*	*Globicatella*	*Leuconostoc*
C16:0	−0.613**	0.784**	0.605*	−0.347	0.722**	0.728**	0.773**
C18:1n-9	0.265	−0.131	−0.227	0.383	−0.239	−0.253	−0.199
C18:2n-6	0.639**	−0.619**	−0.597*	0.584*	−0.682**	−0.699**	−0.676**
C18:3n-3	0.151	−0.339	−0.173	−0.079	−0.229	−0.222	−0.285
C20:4n-6	−0.719**	0.853**	0.698**	−0.482	0.823**	0.833**	0.863**
C20:5n-3	−0.718**	0.852**	0.696**	−0.479	0.822**	0.832**	0.862**
C22:6n-3	−0.719**	0.853**	0.698**	−0.482	0.823**	0.833**	0.863**
EPA + DHA	−0.718**	0.853**	0.697**	−0.48	0.822**	0.833**	0.862**
ΣSFA[a]	−0.659**	0.818**	0.645**	−0.399	0.767**	0.774**	0.814**
ΣMUFA[b]	−0.550*	0.732**	0.547*	−0.279	0.658**	0.661**	0.712**
Σn-3PUFA[c]	−0.160	0.007	0.125	−0.312	0.119	0.132	0.074
Σn-6PUFA[d]	0.625**	−0.583*	−0.572*	0.576*	−0.653**	−0.670**	−0.643**
ΣPUFA[e]	0.619**	−0.789**	−0.610**	0.353	−0.728**	−0.733**	−0.778**

Note: All values are means ± SEM (n = 6). * and * * indicates a significant correlation ($P < 0.05$) and very significant correlation ($P < 0.01$), respectively, by Pearson Correlation analysis. Only the top 7 most abundant (based on relative abundance) bacterial genera which have a significant correlation with individual dietary fatty acid content are shown. C16:0 is the predominant fatty acid of SFA; C18:1n-9 is the predominant fatty acid of MUFA; and C18:2n-6, C18:3n-3, C20:4n-6, C20:5n-3, C22:6n-3 are the predominant fatty acid of PUFA.

[a] ΣSFA is the sum of saturated fatty acids including C6:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C18:0, C22:0, C23:0.

[b] ΣMUFA is the sum of monounsaturated fatty acids including C14:1, C16:1, C18:1, C20:1, C24:1n-9.

[c] Σn-3PUFA is the sum of n-3 polyunsaturated fatty acids including C18:3n-3, C20:3n-3, C20:5n-3 (EPA), C22:6n-3 (DHA).

[d] Σn-6PUFA is the sum of n-6 polyunsaturated fatty acids including C18:2n-6, C20:3n-6, C20:4n-6.

[e] ΣPUFA is the sum of polyunsaturated fatty acids including C18:2n-6, C18:3n-3, C20:3n-6, C20:3n-3, C20:4n-6, C20:5n-3 (EPA), C22:6n-3 (DHA).

and Tenericutes were observed in the intestinal community of golden pompano fed the three diets, with Firmicutes and Proteobacteria being the predominant phyla in the intestine of golden pompano, which is in accordance with previous studies in fish [17,54,55]. Firmicutes was the most abundant phylum in the VF group, while the phylum Tenericutes increased gradually from the VF to VL and VS groups. Previous studies have reported that the phylum Tenericutes belongs to the indigenous intestinal microbiota of fish [23,56], and that plant ingredients in diets could increase the phylum Tenericutes of the intestinal microbiome [57], suggesting that dietary VO might have a more profound effect on the phylum Tenericutes compared to dietary FO in intestine of golden pompano. At genus level, *Mycoplasma*, a genus belongs to Tenericutes phylum, accounted for 63.86%, 47.81% and 8.61% of the abundant microbiota in the VS, VL and VF groups, respectively. *Mycoplasma* (Tenericutes, Mollicutes) are gram-positive bacteria that lack a cell wall, and can parasitize the cellular membrane of eukaryotes. All known *Mycoplasm*a species are considered to be pathogens or parasites [58,59]. However, Holben et al. (2003) reported of a novel *Mycoplasma* phylotype, as a normal inhabitant of the gut of salmonid fishes, which played an unknown role in the health and physiology of fish [60]. Meanwhile, dietary VO was also observed to significantly increase the abundance of genus *Vibrio* and decreased the relative abundance of lactic acid bacteria (LAB) compared with dietary FO. Members of *Vibrio* are ubiquitous and are associated with various marine environments and fish species [61]. *Vibrio* bacteria are important pathogens present in the intestinal flora of marine fish due to infections, commonly causing or producing bacteremia [62,63]. Sun et al. (2018) recently reported that *Vibrio* prefers rich-lipid surroundings [23]. Similarly, previous studies revealed that *Vibrio* have the ability to absorb C18:2n-6 [64,65]. In the present study, it was observed that dietary FO seems to have a more profound effect by promoting the growth of healthy intestinal bacterial community in golden pompano, due to its potency to inhibit pathogenic bacteria and enhance the abundance of probiotics. In addition, correlation analysis showed that long-chain polyunsaturated fatty acids (LC-PUFA), especially EPA and DHA, had a more profound effect on increasing the intestinal probiotics of golden pompano. This is consistent with previous reports, in which a significant increase in both total viable counts and population level of LAB was observed in gut and feces of fish fed 7% 18:3n-3 or 4% LC-PUFA mix, coupled with a lower frequency of LAB found in fish fed with dietary linoleic acid (18:2n-6) [53]. In addition, Mills et al. (2010) reported that n-3 PUFAs of linseed oil and fish oil, especially n-3 LC-PUFA, are recognized as having an anti-inflammatory effect by inhibiting pathogenic bacteria and enhancing the abundance of probiotics [66]. However, the functions of dietary FO in golden pompano intestine still remain unknown due to limited studies of gut microbiota in fish. The present study indicates that dietary soybean oil and linseed oil, when compared with FO, caused an imbalance between commensal bacteria with pathogenic potential and those with potential benefits, which would disrupt intestinal homeostasis and play a role in intestinal nutrient uptake, immunity and mucosal barrier integrity [19,54,67]. And the result was consistent with our feeding trial that the full replacement of FO by the two vegetable oils (especially SO) exerted negative effects on the growth and feed efficiency of the fish (unpublished). Therefore, the results suggest that intestinal homeostasis is beneficial to the immune system and optimal nutrient uptake, which is necessary for normal growth and health of fish.

5. Conclusions

In conclusion, the results from the present study suggest that dietary VO, especially soybean oil, exerts negative effects on intestinal health in golden pompano, including gut microbiota, intestinal digestive functions, immunity and mucosal barrier integrity. Notably, dietary VO greatly modified the composition of the intestinal microbiota community with a significant increase in abundance of potentially pathogenic bacteria, and impaired the normal function of intestine. Moreover, dietary supplementation with soybean oil had a more profound effect on the intestinal microbiome and intestinal health of golden pompano compared to linseed oil. As far as vegetable oils are concerned, linseed oil is more suitable for replacing FO as dietary lipid source compared to soybean oil. The data also reveals that dietary LC-PUFA, especially EPA and DHA, may have a more positive effect on enhancing the intestinal probiotics of golden pompano.

Acknowledgements

This work was financially supported by the Research Projects from the National Natural Science Foundation of China (No. 31873040), China Agriculture Research System (CARS-47) and Department of Education of Guangdong Province (2016KTSCX037), as well as Natural Science Foundation of Guangdong Province (2018A030313910).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.060.

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