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Research Article

Effect of Vitamin A Supplementation on Growth Performance, Lipid Deposition, Antioxidant Ability, and Immunity in Juvenile Chinese Mitten Crab *Eriocheir sinensis* Fed Diet with Fish Oil Totally Replaced by Palm Oil

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This research evaluated the protective effect of vitamin A (VA) on the adverse effect of fish oil (FO) substitution with palm oil (PO) in an economical crab *Eriocheir sinensis*. Three diets of FO, PO, and PO + 8000 IU/kg VA as the main lipid sources were fed to crabs, respectively, for 8 weeks. Compared to crabs fed FO diet, crabs fed PO diet showed reduced hemolymph VA concentration, feed utilization efficiency, and growth performance. Besides, crabs fed PO diet showed elevated lipid content in hepatopancreas and body and triglyceride content in hepatopancreas, leading to decreased antioxidant enzyme and immune parameters activities from biochemical analysis, enzymatic determination, and quantitative polymerase chain reaction. In contrast, compared to crabs fed PO only, VA supplementation in PO improved the growth performance and utilization of fatty acids and reduced lipid deposition in the hepatopancreas. In addition, VA supplementation suppressed gene expression related to triglyceride synthesis (*dgat1*) and positively affected gene expression related to lipid catabolism (*cpt1a*, *cpt1b*, *cpt2*, and *caat*). Furthermore, VA supplementation upregulated antioxidant genes (*CuZnSOD* and *CAT*) through downregulating gene expression of upstream regulator *Keap1*. Furthermore, VA supplementation upregulated immune genes (*Lzm* and *proPO*) expression and reduced proinflammatory genes (*LITAF*, *ADAM17*, and *IL-16*) expression related to *Toll2*/*MyD88*/*Relish* signaling pathway. This study shows the necessity of VA addition in the feed with FO totally replaced by PO because it can relieve PO's adverse effects and improve the growth of crabs.

1. Introduction

Lipids are essential nutrients in the diets of aquatic animals since they provide fatty acids and serve as the constituent of biological membranes, coenzyme factors, precursors of hormones and vitamins, and lipid-soluble vitamin carriers [1, 2]. Due to the limited synthesis capacity of polyunsaturated fatty acids (PUFA) in fish and crustaceans, it is necessary to supply exogenous lipids in the diet [3, 4]. Fish oil (FO) is traditionally used in aquaculture feed because it can provide abundant PUFAs required for normal growth, molting, stress resistance, and bodily functions of aquatic animals [5–7]. However, the global shortage of FO production makes it urgent to exploit alternative lipid sources to replace FO for the sustainable development of aquaculture [8].

Researchers have paid attention to seeking alternative lipid sources to replace FO, and vegetable oil has been demonstrated to be the best candidate for FO in feed due to its high availability, reasonable price, and composition of fatty acids [8]. Up to now, the uses of different vegetable oil sources such as perilla oil, safflower seed oil, olive oil, canola oil, oil-tea camellia seed oil, linseed oil, soybean oil, rapeseed oil, sunflower oil, peanut oil, corn oil, rice oil, coconut oil, and palm oil (PO) have been studied in various fish and crustacean species [5, 9–19]. Among all vegetable oil sources, PO has a lower price and higher productivity relative to other vegetable oils, and the demand for renewable energy has made it one of the most traded oils in the world [20]. The PO is rich in saturated fatty acids (SFAs), C16:0, and C18:1n-9 (OA), and it is not easy to rancidity [17]. Compared to FO, PO lacks n-3 highly saturated fatty acids (n-3 HUFA) such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) and leads to the imbalance of n-3/n-6 polyunsaturated fatty acid (PUFA) ratio, which is vital for normal growth and physiological function of animals [21–23]. Therefore, excessive PO in the diet can impair the growth of black tiger shrimp *Penaeus monodon* and swimming crab, *Portunus trituberculatus* [10, 14]. Complete substitution of FO with PO can cause growth suppression and excessive lipid deposition in grouper, *Epinephelus coioides* [17], Chu's croaker, *Nibea coibor* [24], and tiger puffer, *Takifugu rubripes* [25]. Besides, due to the imbalance of fatty acid profile (especially n-3 and n-6 PUFA), the substitution of FO with vegetable oil could induce inflammation and impair antioxidant capacity and immunity [6, 26–30]. To better develop global aquaculture, it is urgent to explore ways to mitigate the negative effect of FO replacement by vegetable oil, improving vegetable oil utilization.

Except for combining different oil sources to balance the fatty acid composition, there is an upward trend in using exogenous additives such as L-carnitine, chenodeoxycholic acid, soy lecithin, sesamin, and antioxidants to relieve the negative effect of dietary vegetable oil [14, 31–34]. Attention has been paid to the nutrient substance content discrepancy between FO and vegetable oil. A high percentage of plant ingredients can usually cause an unbalanced vitamin supply [35]. Vitamin A (VA, including retinyl esters, retinal, retinol, and retinoic acid in a broad sense), an essential nutrient, could not be synthesized in the body and should be obtained from animal diets mainly as retinyl esters or plant-derived precursors as β-carotene [36, 37]. FO is rich in VA, while the substitution of FO with vegetable oil would result in VA deficiency [38, 39]. Although some aquatic animals could convert β-carotene to VA, the conversion efficiency is very low and difficult to satisfy the requirements [40]. Therefore, there is a need to supplement exogenous dietary VA with FO largely substituted by PO. Furthermore, VA is a potential regulatory factor to positively affect lipid metabolism with enhanced lipid catabolism, leading to a lipid reduction effect [41–44]. It has been well recognized that a proper VA level could improve growth, antioxidant ability, and immunity for aquatic animals [45–47]. Thus, supplementation of VA in PO might be a promising regulatory factor to alleviate the negative effect of a high percentage of PO. Therefore, there is a need to determine the necessity and protective effect of exogenous dietary VA supplementation in diets for a FO total substitution with PO.

Chinese mitten crab *Eriocheir sinensis* is a famous crustacean species and popular with public consumption because of its high nutritional quality [48]. In China, the production of *E. sinensis* exceeded 770,000 tons in 2020 [49]. In the study of crabs' nutrition physiology, FO (about 30 g/kg) is widely used in feed formulation, but there is a need to find a substitute [50–52]. In practice, the supply of small trash fish cannot meet the increasing demand and may cause environmental pollution to hinder the development of *E. sinensis* breeding industry [53]. Thus, the research to improve the utilization of vegetable oils can be beneficial to the sustainable development of aquaculture and cost-effective feed. Furthermore, the previous studies have demonstrated that supplementing 8000 IU/kg dietary VA level based on a diet using vegetable oil as the main lipid source could lead to the best growth in *E. sinensis*, which provided a foundation for the present study [41]. Thus, this study aimed to investigate the influence of total replacement of FO with PO and VA supplementation to PO diet on growth performance, lipid metabolism, and immune response of juvenile *E. sinensis*.

2. Materials and Methods

2.1. Experimental Diets. Three iso-nitrogenous (crude protein-40% approximately) and iso-lipidic (crude lipids-8% approximately) experimental diets were formulated by replacing all the FO with PO and further supplementing 8000 IU/kg vitamin A to the PO diet (PO + VA) (Table 1). The specific procedures of preparation and processing of diets referred to the previous study in our lab [41].

2.2. Animals and Farming Method. The crabs were from a commercial breeding base in Chong Ming (Shanghai, China). The feeding experiment (8 weeks) was implemented at Zhejiang Freshwater Fisheries Research Institute, Hu Zhou (Zhejiang, China). Before the feeding trial, crab juveniles were raised and fed (self-made vitamin A-free feeds, crude protein-40%, crude lipid-7%) in square pools ($2 \times 1.7 \times 0.6$ m) for one week to acclimate the experimental conditions. After being fasted for 24 h, 600 crabs of a homogenous size and intact appendages (average weight: 0.69 ± 0.01 g, mean weight ± SEM, standard error of the mean) were randomly allocated into 15 independent tanks (300 L, three groups) corresponding to quintuplicate tanks (40 crabs per tank) each group. Four clean and sterile tiles and 30 plastic pipes per tank were applied to avoid animal fighting. Crabs were fed 4% of their body weight three times every day for 8 weeks (8 : 00, 14 : 30, and 21 : 30) following the natural day and night cycle. The faeces were removed by a siphon tube, and 2/3 of the total water was replaced with filtered water every day. Total consumptive and superfluous feeds were recorded to calculate the feed conversion ratio (FCR). The daily environmental conditions were

Table 1: Raw materials of the feed formula (g/kg dry basis) and the diet proximate composition (% dry weight).

Material	Different diets		
	FO	PO	PO + VA
Vitamin-free casein[1]	360	360	360
Gelatin	90	90	90
Corn starch	260	260	260
Soybean lecithin oil	10	10	10
Palm oil		55	55
Fish oil	55		
Cholesterol	5	5	5
Choline chloride	5	5	5
Vitamix mix[1]	40	40	40
Mineral mix[2]	20	20	20
Calcium dihydrogen phosphate	10	10	10
Attractant	30	30	30
Antioxidant	1	1	1
Carboxy methyl cellulose	20	20	20
Microcrystalline cellulose	94	94	82
VA dilution (5000 IU/g)[3]	0	0	12
Total (g)	1000	1000	1000
Proximate composition (%)			
Moisture	11.93	11.98	11.01
Crude protein	40.77	40.48	39.89
Crude lipid	7.77	7.64	7.79
Crude ash	4.66	4.56	4.52
Detected VA content (IU/kg)	1711	ND[4]	8506

[1]Vitamin mix (g per kg premix): inositol, 10; ascorbic acid, 5 g; tocopheryl acetate, 5; niacin, 3; D-calcium pantothenate, 3; pyridoxine hydrochloride, 2.25; thiamine mononitrate, 1.5; riboflavin, 0.625; menadione, 0.5; folic acid, 0.25; cyanocobalamin, 0.1; cholecalciferol, 0.075; biotin, 0.05. α-cellulose is used as fillers. [2]Mineral mix (g per kg premix): $Ca(H_2PO4)_2$, 265; KH_2PO_4, 215; $CaCO_3$, 105; $NaH_2PO_4{\cdot}H_2O$, 100; $MgSO_4{\cdot}7H_2O$, 100; KCl, 28; Fe-citrate, 10; $ZnSO_4{\cdot}7H_2O$, 4.76 g; $MnSO_4{\cdot}6H_2O$, 1.43; $CoCl_2{\cdot}6H_2O$, 1.4; $AlCl_3{\cdot}H_2O$, 0.24; KI, 0.23; $CuCl_2{\cdot}2H_2O$, 0.15. α-Cellulose is used as fillers. [3]Retinyl esters (Minsheng Biotechnology Co. Ltd., Zhejiang, China) diluted to 5000 IU/g by rice bran powder before addition. [4]Under the detection range.

temperature 23-27°C, pH 7.3 to 8.4, dissolved O_2 > 7.0 mg/L, and ammonia nitrogen < 0.05 mg/L.

2.3. Sampling Method. At the termination of the growth trial, crabs were counted to calculate the survival rate (SR) after being fasted for 24 h. Then, the animals were weighed to obtain the final body weight (FBW), specific growth rate (SGR), and weight gain (WG). Five crabs per tank were kept at a -20°C refrigerator for body proximate composition analysis. The hepatopancreas of three crabs per tank was weighed to determine the hepatosomatic index (HSI) value, and then, the hepatopancreases were mixed for total lipid content and fatty acid composition analysis. The mixed haemolymph per tank was collected from six crabs, and the pretreatment method of the haemolymph prior to biochemical detection was referred to Bu et al., 2020 [54]. The hepatopancreas from four crabs per tank was pooled and then immediately frozen in a nitrogen canister and finally transferred to the -80°C refrigerator for enzyme activity detection and RNA isolation. The homogenate of hepatopancreas was made before enzyme activities determination according to the previous study in our lab [55]. All of the operations were conducted on ice cubes to reduce crab activity.

2.4. Growth-Related Parameter Calculations. $\mathrm{SR}\,(\%) = 100 \times N_t / N_0$.

$\mathrm{WG}\,(\%) = 100 \times [W_t\,(\mathrm{g}) - W_0\,(\mathrm{g})] / W_0\,(\mathrm{g})$.

$\mathrm{SGR}\,(\%/\mathrm{day}) = 100 \times [\ln W_t\,(\mathrm{g}) - \ln W_0\,(\mathrm{g})]/\mathrm{days}$.

$\mathrm{HSI}\,(\%) = 100 \times \text{hepatopancreas weight}\,(\mathrm{g})/\text{body weight}\,(\mathrm{g})$.

$\mathrm{FCR} = \text{total dry feed intake}\,(\mathrm{g}) / [W_t\,(\mathrm{g}) - W_0\,(\mathrm{g})]$.

Where N_t is the final fish number, N_0 is the initial fish number, W_t is the final body weight, and W_0 is initial body weight.

2.5. Composition and Fatty Acid Analysis. The standard methods [56] were applied to detect the biochemical composition (moisture, crude protein, and ash) of feeds and the whole body of crabs. The samples were dried at 105°C in the oven up to a constant weight. Crude protein was measured through the method of Kjeldahl ($N \times 6.25$) (8200, Kjeltec, Foss, Sweden). Samples were first carbonized and then made into ash in a muffle furnace at 550°C.

The chloroform/methanol (2 : 1, *v*/*v*) mixture and KCl solution (0.37 mol/L) were used to get the total lipids in the whole body, hepatopancreas, or feed [55]. Hepatopancreas or feed lipid methylation was obtained using 0.5 mol KOH per litre methanol at 65°C for 1 hr and 14% BF3 in methanol at 75°C for 30 min. The fatty acid methyl esters (1.0 μL) were dissolved in n-hexane (GC residue analysis, CNW, China) and finally analyzed by gas chromatography-mass spectrometry analysis (GCMS-QP2010 SE, Shimadzu Co., Kyoto, Japan) with gas chromatographic column (SH-Rt-2560, 0.25 mm ID, 0.20 μm df, 100 m, Shimadzu, USA). Speed of carrier gas helium: 1.7 mL/min. Injector and flame ionization detector temperatures: 250°C and 200°C, respectively. The programs: 120°C for 1 min, 240°C for 30 min, and the total measurement time was 61 min for each sample. The identified fatty acid was expressed as the area percentage of total fatty acids. Fatty acid profiles of different diets are shown in Table 2.

The high-performance liquid chromatography (HPLC) method determined the vitamin A in feeds and hemolymph [57]. The specific parameters and procedure referred to our previous work [58].

2.6. Biochemical, Immune, and Antioxidant Indices. Commercial kits from Jiancheng Biological Engineering Institute (Nanjing, China) were used for the following indices detection: aspartate aminotransferase (AST, NO. C010-2-1), alanine aminotransferase (ALT, NO. C009-2-1), alkaline phosphatase (AKP, NO. A059-2-1), and acid phosphatase (ACP, NO. A060-2-1) in hemolymph, triglycerides (TG, NO. F001-1-1), and malondialdehyde (MDA, NO. A003-1-1) content, superoxide dismutase (SOD, NO. A001-3-1), glutathione peroxidase (GPx, NO. A005-1-1) activities, and

Table 2: The fatty acid composition of different experimental diets (% total fatty acids).

Fatty acids	Different diets		
	FO	PO	PO + VA
C12:0	1.17	0.83	1.04
C14:0	9.90	3.66	3.44
C16:0	28.73	29.05	29.13
C18:0	8.27	5.84	5.36
C20:0	0.36	0.26	0.24
C14:1n-9	0.28	0.16	0.17
C16:1n-7	7.82	1.27	0.98
C18:1n-9 (OA)	19.91	38.35	38.86
C20:1n-9	1.32	0.34	0.30
C18:2n-6 (LA)	7.75	17.99	17.79
C18:2n-7	0.20	0.25	0.48
C18:3n-3 (LNA)	2.11	2.00	2.21
C20:2n-6	0.13	ND	ND
C20:4n-6 (ARA)	0.93	ND	ND
C20:5n-3 (EPA)	5.84	ND	ND
C22:5n-3 (DPA)	0.52	ND	ND
C22:5n-6	0.28	ND	ND
C22:6n-3 (DHA)	4.50	ND	ND
ΣSFA	48.42	39.64	39.21
ΣMUFA	29.32	40.12	40.31
ΣPUFA	22.26	20.24	20.48
Σn-6 PUFA	9.08	17.99	17.79
Σn-3 PUFA	12.97	2.00	2.21
SFA: PUFA	2.18	1.96	1.91
Σn-3: n-6 PUFA	1.43	0.11	0.12

FO: fish oil; PO: palm oil; PO + VA: palm oil diet with vitamin A addition; OA: oleic acid; LA: linoleic acid; LNA: linolenic; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acid; ND: not detected. The fatty acids whose content was minor or was not detected were not listed. SFA: C12:0, C14:0, C16:0, C18:0, C20:0. MUFA: C14:1n-9, C16:1n-7, C18:1n-9, C20:1n-9. PUFA: C18:2n-6, C18:2n-7, C18:3n-3, C20:2n-6, C20:4n-6, C20:5n-3, C22:5n-3, C22:5n-6, and C22:6n-3.

total antioxidation capability (T-AOC, NO. A015-2-1) in the hepatopancreas. According to Ashida and Masaaki, hemolymph phenoloxidase (PO) was measured using L-dopa [59]. In addition, haemocyanin concentrations were detected through spectrophotometry at 335 nm [54].

2.7. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). The amplification efficiency of primers has been verified based on the formula $E = 10^{(1/\text{Slope})} - 1$, and they were in the range of 90 to 105. β-Actin and S27 were used as the internal standard (Table 3). Kits from Vazyme Biotech Co were used for RNA isolation, inverse transcription, and *RT-qPCR*. Total RNA extraction was realized using Trizol, following the manufacturer's protocol. The concentration and integrity of RNA were guaranteed by Agilent 2100 Bio-analyzer (Agilent Technologies, USA). RNase-free agarose gel electrophoresis was applied to check the RNA quality. Then genomic DNA was removed, and reverse transcription was done to get the cDNA product for *RT-qPCR* using kits (Cat. No. R323-01). The process of *RT-qPCR*, including the reaction system and the procedure, was the same as previous work in crabs [41]. The method of $2^{-\Delta\Delta Ct}$ was applied to calculate the relative mRNA expression level [60].

2.8. Statistical Analysis. The software SPSS 20.0 (Chicago, IL, USA) was used to deal with the data. Variance homogeneity and distribution normality were checked before Student's *t* -test in pairs. It was considered to be significant if statistical P value < 0.05, and extremely significant at P value < 0.01. Data were shown as means ± SEM.

3. Results

3.1. Growth Performance and Feed Utilization. Crabs fed the FO diet showed significantly higher values of FBW, WG, and SGR compared to crabs fed the PO ($P < 0.01$) and PO + VA diets ($P < 0.05$, Figures 1(a)–1(c)). Whereas vitamin A addition in the PO diet raised FBW, WG, and SGR compared to crabs fed PO diet alone ($P < 0.05$). HSI ($P < 0.05$) and FCR ($P < 0.01$) in crabs fed the PO diet were significantly higher than those fed other diets (Figures 1(e) and 1(f)). There was no significant difference in HSI and FCR between crabs fed FO and PO + VA diets ($P > 0.05$). Different diets did not affect the SR of crabs ($P > 0.05$, Figure 1(d)).

3.2. Body and Hepatopancreas Lipid Deposition. Crabs fed diets of PO ($P < 0.05$) and PO + VA ($P < 0.01$) prominently reduced the crude protein content in the whole body of crabs relative to those fed a diet of FO (Figure 2(b)). On the other hand, the diet of PO fed to crabs increased the total lipid content in the whole body compared to the diet of FO ($P < 0.05$, Figure 2(c)). Although no significant difference was observed, crabs fed PO + VA showed lower total lipid content in the whole body than those fed PO ($P > 0.05$). Different diets did not affect crabs' moisture and ash content ($P > 0.05$, Figures 2(a) and 2(d)).

Crabs fed PO showed higher total lipid content and TG content in the hepatopancreas than those fed other diets ($P < 0.05$, Figures 2(e) and 2(f)). Whereas crabs fed PO + VA reduced the total lipid content and TG content in the hepatopancreas relative to those fed PO diet ($P < 0.05$).

Hemolymph VA content showed the lowest in crabs fed PO diet, whereas it appeared to be a conspicuous higher value in crabs fed PO + VA relative to diet PO ($P < 0.05$, Figure 2(g)).

3.3. Fatty Acid Profiles of Hepatopancreas. Crabs fed diets of PO and PO + VA showed lower ΣSFA content and SFA, including C14:0, C16:0, C18:0, C20:0, and C22:0, compared to those fed FO diet ($P < 0.01$, Figures 3(a) and 3(c)). Crabs fed PO + VA decreased the C12:0 ($P > 0.05$) and C16:0 ($P < 0.01$) content in hepatopancreas compared to those fed PO diet alone.

C14:1n-9, C18:1n-7, C18:1n-9, and C20:1n-9 in the hepatopancreas of crabs fed PO, and PO + VA was

Table 3: Information of gene primers used for qPCR experiment.

Genes	Sequence (5′-3′)	Reference
dgat1	(F)CGAGCACATAACACAAGCGG (R)AACCAGACGACCACGAGAAC	GenBank: MF497735.1
cpt1a	(F)CATCTGGACACCCACCTCCA (R)ATCTCCTCACCCGGCACTCT	GenBank: MH037158.1
cpt1b	(F)GGCATTCTCCTTTGCCATCAC (R)ACACCACACCGCACATTGTTC	GenBank: MH037159.1
cpt2	(F)AGCAGGCAGTGGCTCAGTTTA (R)AAGGCAAGGAAGGGGTTGTAG	GenBank: MH037160.1
caat	(F)CATCAAGAGCCAGGAGCCCA (R)CTTCAACAGCAGCCCGCAAA	Lin et al. [61]
GPx	(F) GACTACACCCCAGTGTGCACCA (R) TGATCCAGCCATTGTGATCCTC	GenBank: FJ617305.1
CAT	(F) ATCCTGCTGCAGGACATCCAA (R) TGATGTCGTGGGTGACCTCAAA	GenBank: GU361618.1
CuZnSOD	(F) ATGAGTAAGACCTTTGCCTA (R) TCCGTCAGTCCATAGATAAC	GenBank: JF815375.1
Keap1	(F) GGCGGTGGTGAACAGACTTA (R) CACAAGGGTCCACTGGTTGT	Qi et al. [62]
Lzm	(F) TACTGGTACTGGTACGACGGCGGAGAG (R) CATCATCGGCGGTCACGTATCGGTTCA	GenBank: JN416111.1
proPO	(F) CCATGTCATCATTGCAGCGG (R) TGTACTTGTGCCAGCGGTAG	GenBank: EF493829.1
ADAM17	(F) GATGTCCGCAACCTGCTAGA (R) CAGGATGCCCCCTTCAAACT	GenBank: KC007532.1
LITAF	(F) ATCAGCTCCCCCACCCTATG (R) GTTGTTGGAGCAGCACxCTTG	GenBank: KF892539.1
IL-16	(F) AGAGGTTGTTCTTGTGCTGTCC (R) ACGAGGGTAATGGTGAATGGAG	GenBank: MG182159.1
Toll2	(F) CATACCAGGACGACGAAC (R) AGACATTGAGCGAGGAGA	GenBank: KC011816.1
MyD88	(F) GCCATCGCAGTCGCCAAGTT (R) GGCATCCTGTTCATCCAGTTCTGAC	GenBank: KC019316.1
Relish	(F) ACAACAGCCGAAGGAGTCAG (R) AGACACTCAGCAGCACTCAC	GenBank: GQ871279.1
β-Actin	(F)TCGTGCGAGACATCAAGGAAA (R)AGGAAGGAAGGCTGGAAGAGTG	Lin et al. [61]
S27	(F)CCCCCAAGAAGATCAAGCACA (R)CAGATGGCAGCGACCACAGTA	Lin et al. [61]

Notes: *dgat1*: diacylglycerol acyltransferase 1; *cpt*: carnitine palmitoyltransferase; *caat*: carnitine acetyltransferase; *GPx*: glutathione peroxidase; *CAT*: catalase; *CuZnSOD*: Cu/Zn-superoxide dismutase; *Keap1*: Kelch-like ECH-associated protein1; *Lzm*: lysozyme; proPO: prophenoloxdiase; *ADAM17*: a disintegrin and metalloproteinase 17; *LITAF*: lipopolysaccharide-induced *TNFα* factor; *IL-16*: interleukin 16; *MyD88*: myeloid differentiation factor 88; *S27*, ubiquitin/ribosomal S27 fusion protein; F: forward; R: reverse.

significantly lower than those fed a diet of FO ($P < 0.01$, Figure 3(a)). Crabs fed diets of PO showed higher C18:1n-9 content in hepatopancreas than those fed FO ($P < 0.05$). Whereas crabs fed PO + VA showed decreased C18:1n-9 content in hepatopancreas relative to those fed PO diet ($P < 0.05$). ΣMUFA in the hepatopancreas of crabs fed FO diet was significantly lower than those fed other diets ($P < 0.01$, Figure 3(c)).

Crabs fed PO or PO + VA showed significantly higher Σn-6 PUFA and C18:2n-6 levels in hepatopancreas than those fed FO diet ($P < 0.01$, Figures 3(b) and 3(c)) and showed lower levels of Σn-3 PUFA, C20:4n-6, C20:5n-3, C22:5n-3, and C22:6n-3 in hepatopancreas than those fed FO diet ($P < 0.01$). A higher ratio of Σn-3/n-6 PUFA in hepatopancreas and a lower ratio of SFA/PUFA were found in crabs fed FO diet relative to crabs fed other diets ($P < 0.01$).

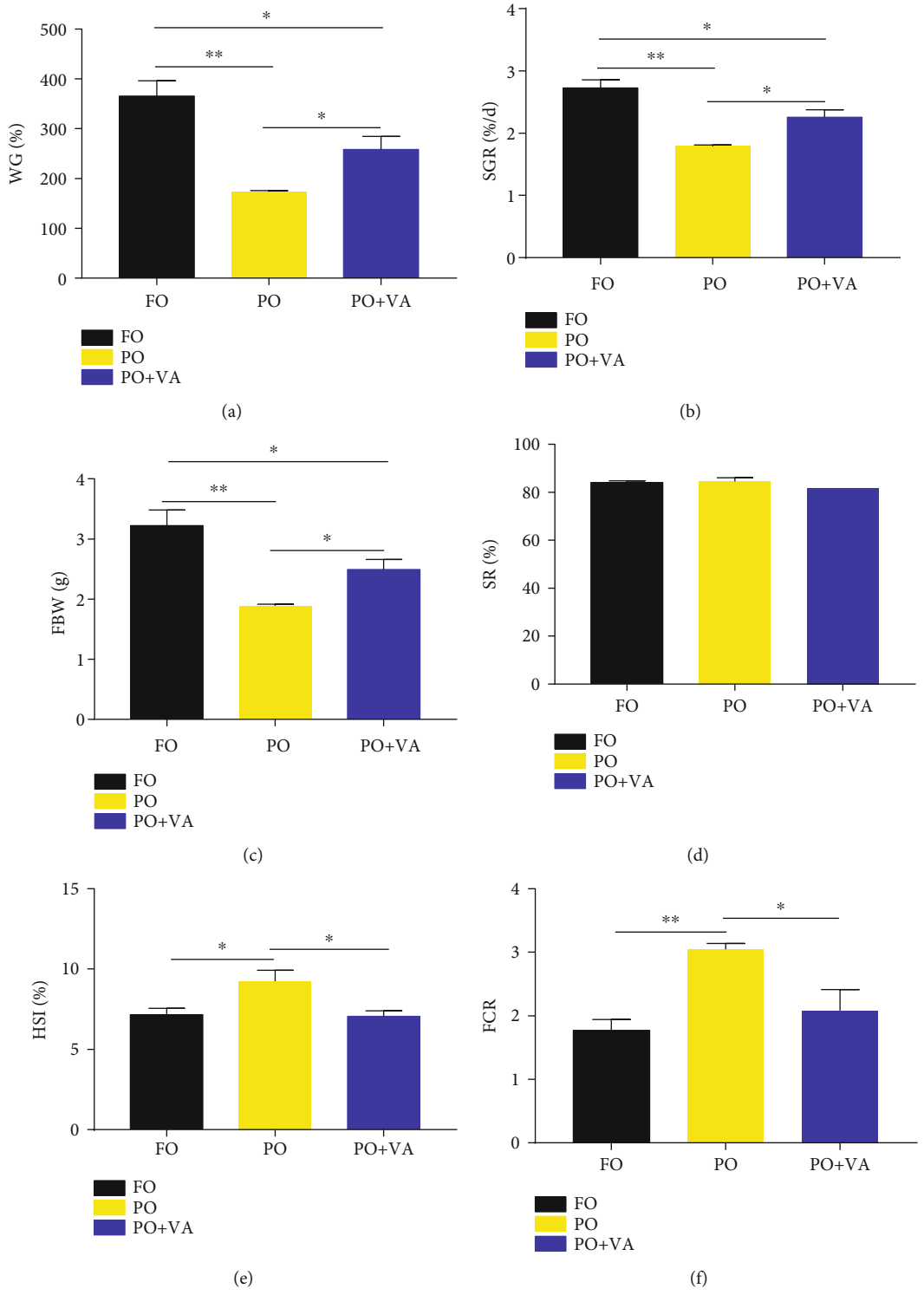


Figure 1: Growth performance and feed utilization of crabs feed different diets. Data represent means ± S.E.M. ($n = 5$). Asterisk (∗) represents a significant difference of $P < 0.05$. Double asterisks (∗∗) represent $P < 0.01$. SR: survival rate; FBW: final body weight; SGR: specific growth rate; WG: weight gain; FCR: feed conversion ratio; HSI: hepatosomatic index; FO (black color): fish oil; PO (red color): palm oil; PO + VA (blue color): palm oil diet with vitamin A addition.

Compared to crabs fed the PO diet, crabs fed PO + VA diet showed a significantly higher level of C18:3n-3, C22:5n-3, and Σn-3 PUFA in hepatopancreas ($P < 0.05$). VA addition in the PO did not affect ΣSFA, ΣMUFA, ΣPUFA, and Σn-6 PUFA in crab hepatopancreas compared to those fed PO only ($P > 0.05$).

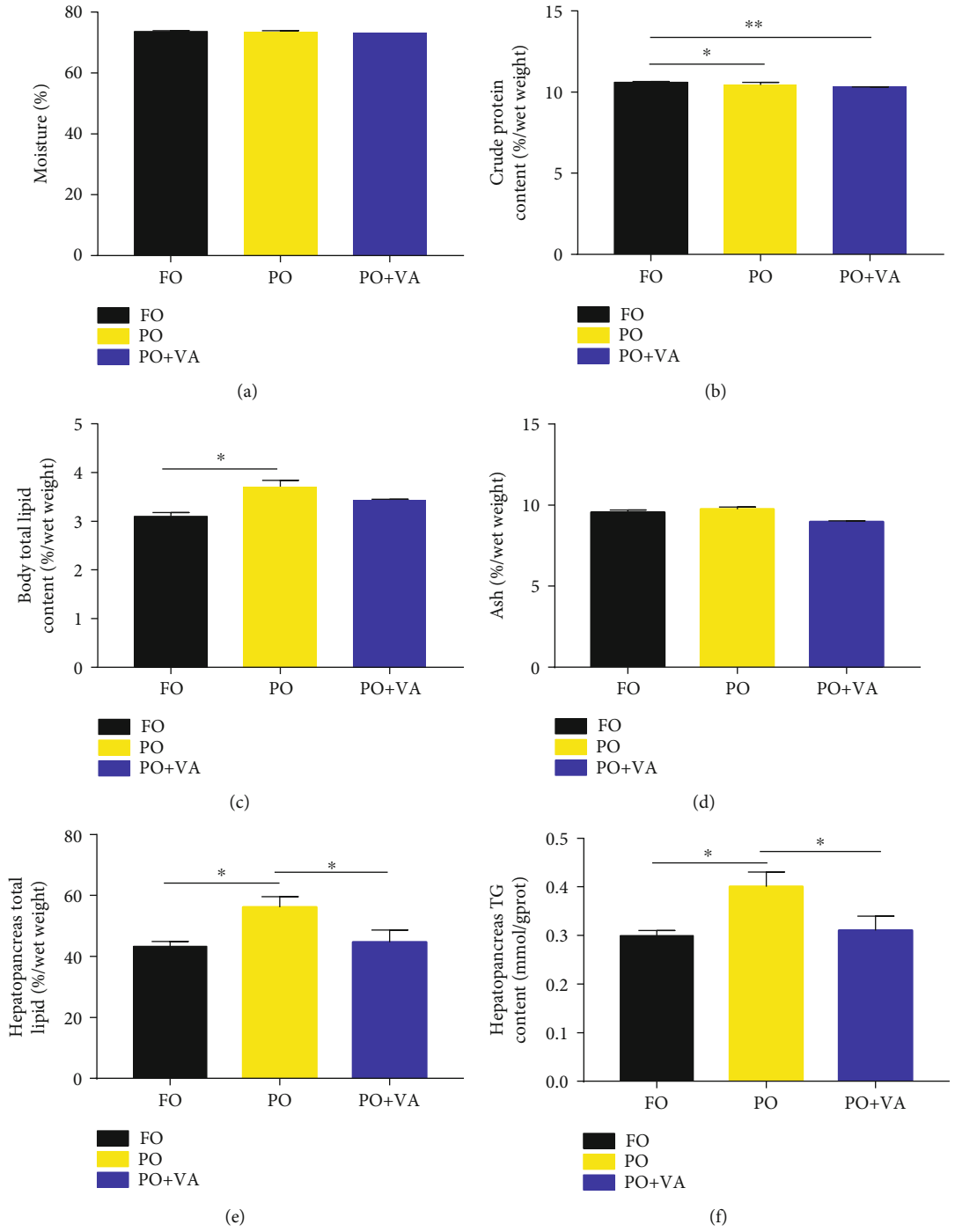


Figure 2: Continued.

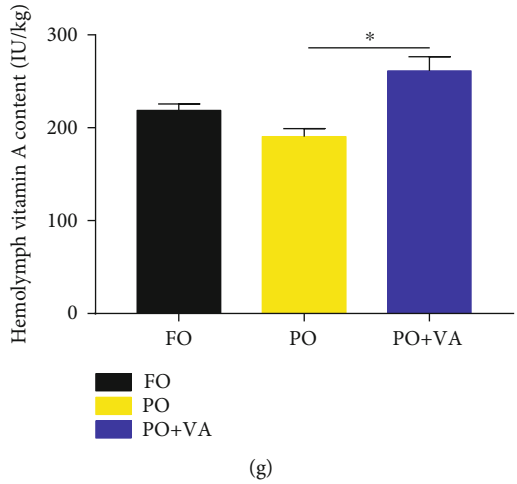


Figure 2: Whole-body composition, lipid content in hepatopancreas, biochemical indices in hepatopancreas and hemolymph of crabs feed different diets. Data represent means ± S.E.M. ($n = 5$). Asterisk (∗) represents a significant difference of $P < 0.05$. Double asterisks (∗∗) represent $P < 0.01$. TG: triglycerides; FO (black color): fish oil; PO (red color): palm oil; PO + VA (blue color): palm oil diet with VA addition.

3.4. Lipid Metabolism-Related Gene Expression. There were higher gene expressions of *dgat1* in the hepatopancreas of crabs fed PO diet relative to those fed FO diet ($P < 0.01$, Figure 4). On the other hand, a lower mRNA level of *dgat1* in hepatopancreas was found in crabs fed diet PO + VA compared with those fed diet PO ($P < 0.05$).

Crabs fed FO showed the lowest mRNA level of genes *cpt1a*, *cpt1b*, *caat*, and *cpt2* in hepatopancreas. The mRNA level of *cpt1a* ($P > 0.05$), *cpt1b* ($P < 0.05$), *caat*, and *cpt2* ($P < 0.01$) in hepatopancreas of crabs fed diet PO were higher than those fed FO diet. Besides, there were higher gene expression of *cpt1a* ($P > 0.05$), *cpt1b*, *caat*, and *cpt2* ($P < 0.05$) in crabs fed PO + VA diet relative to those fed PO alone.

3.5. Antioxidant Capacity and Hepatopancreas Health. There were significantly higher values of the MDA content ($P < 0.01$) and activities of SOD ($P < 0.05$) and GPx ($P < 0.01$) in the hepatopancreas of crabs fed FO relative to those fed PO (Figures 5(a)–5(c)). However, VA addition in PO significantly increased the activities of SOD and GPx ($P < 0.05$) and reduced the MDA content ($P < 0.01$) in the hepatopancreas of crabs compared with those fed PO only. T-AOC in the hepatopancreas was not affected by different diets ($P > 0.05$, Figure 5(d)), whereas crabs fed PO showed a lower value than those fed FO. VA addition in the PO raised T-AOC level in the hepatopancreas of crabs relative to those fed PO alone.

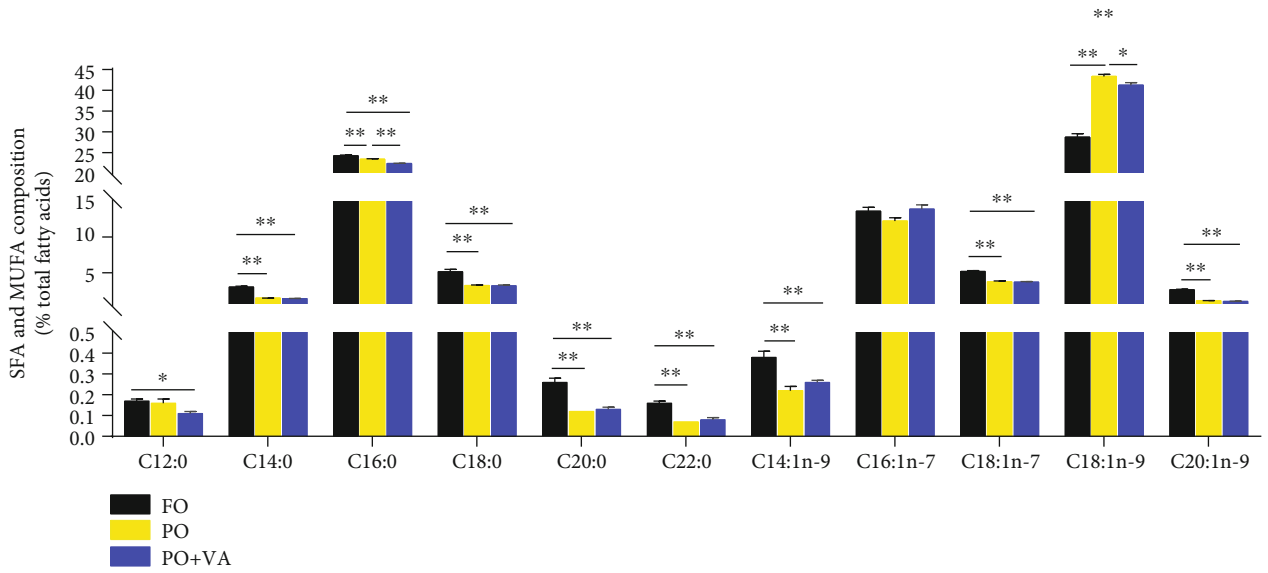
Crabs fed diets of PO prominently increased the hemolymph AST and ALT levels relative to those fed FO ($P < 0.05$, Figures 5(e) and 5(f)). Conversely, crabs fed PO + VA showed a lower hemolymph AST level than those fed PO diet, though no significant difference happened ($P > 0.05$).

3.6. Hepatopancreas Immunity Parameters. Crabs fed FO showed significantly higher hemolymph AKP and ACP activities than those fed PO ($P < 0.05$, Figures 6(a) and 6(b)). Crabs fed PO + VA showed higher AKP ($P > 0.05$) and ACP activities ($P < 0.05$) relative to those fed PO. The activity of Po in hemolymph was not affected by different diets, whereas it showed the lowest in crabs fed PO and was raised by VA addition (Figure 6(c)). Hemocyanin content showed the lowest in crabs fed PO and was significantly increased by VA addition in PO ($P < 0.05$, Figure 6(d)).

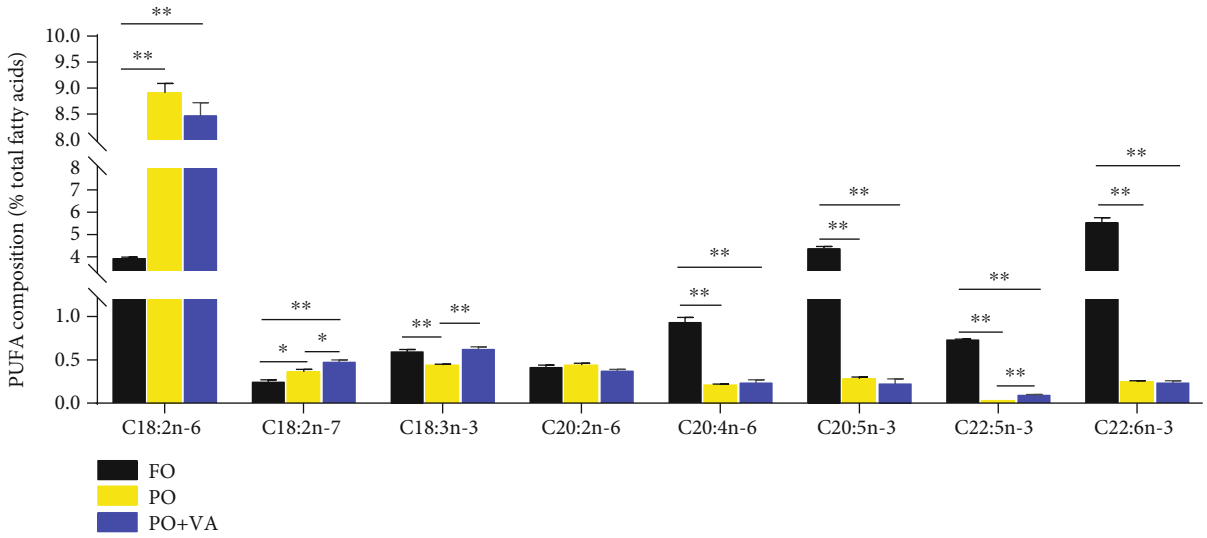
3.7. Antioxidant and Immunity-Related Gene Expression. Crabs fed a PO diet showed lower gene expression of *CuZnSOD*, *GPx*, and *CAT* in the hepatopancreas than those fed FO (Figure 7(a)). Whereas the expression of *CuZnSOD* ($P < 0.01$) and *CAT* ($P < 0.05$) in hepatopancreas was significantly increased by PO + VA relative to diet PO. Higher gene expression of GPx was also found in crabs fed PO + VA relative to diet PO ($P > 0.05$). In addition, crabs fed the PO diet showed a significantly higher mRNA level of *Keap1* gene than crabs fed the FO diet, whereas crabs fed the PO + VA diet reduced the mRNA level of *Keap1* relative to those fed the PO diet ($P < 0.05$).

The gene expression of *Lzm* and *proPO* in crabs fed the PO diet was lower than those fed the FO diet ($P < 0.05$, Figure 7(b)). Whereas crabs fed the PO + VA diet showed significant higher gene expression of *Lzm* ($P < 0.05$) and *proPO* ($P < 0.01$) relative to those fed the PO diet. There was a significantly higher gene expression level of *ADAM17*, *LITAF*, and *IL-16* in the hepatopancreas of crabs fed the PO diet than those fed the FO diet ($P < 0.01$). Nevertheless, lower mRNA levels of *ADAM17* and *IL-16* ($P < 0.05$) and *LITAF* ($P < 0.01$) were observed in the hepatopancreas of crabs fed PO + VA diet versus PO diet.

There were higher gene expression of *MyD88* ($P > 0.05$), *Toll2*, and *Relish* ($P < 0.01$) in the hepatopancreas of crabs fed the PO diet compared to those fed FO diets (Figure 7(c)). Whereas crabs fed PO + VA diet showed decreased gene expression of *MyD88*, *Toll2* ($P > 0.05$), and *Relish* ($P < 0.05$) relative to those fed PO diet.

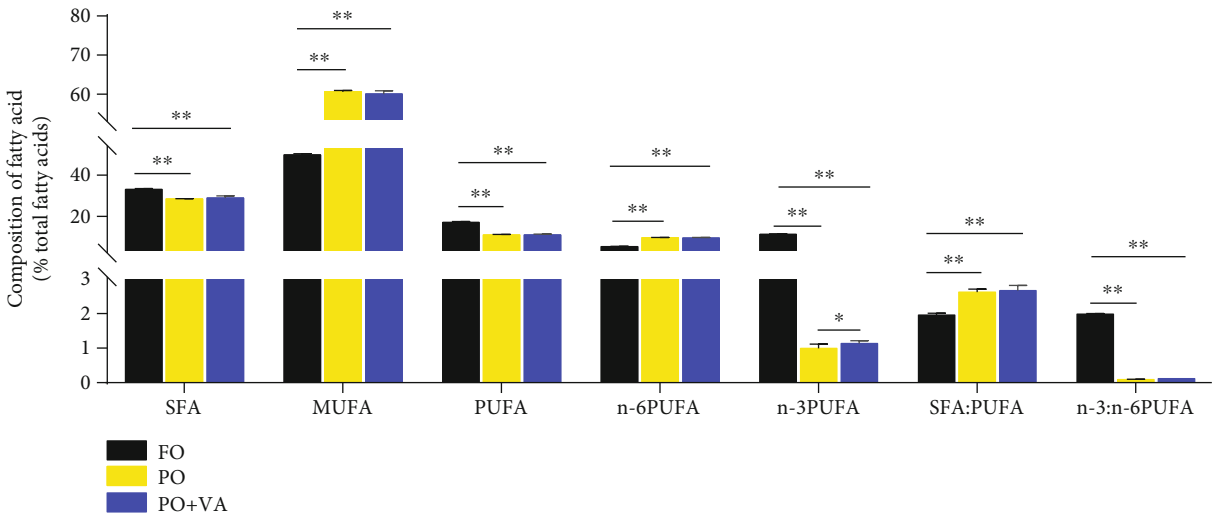


(a)



(b)

Figure 3: Continued.



(c)

Figure 3: The fatty acid composition of the hepatopancreas in crabs fed different experimental diets (% total fatty acids). Data represent means ± S.E.M. ($n = 5$). Asterisk (∗) represents a significant difference of $P < 0.05$. Double asterisks (∗∗) represent $P < 0.01$. FO (black color): fish oil; PO (red color): palm oil; PO + VA (blue color): palm oil diet with vitamin A addition; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acid; ND: not detected. The fatty acids whose content was minor or was not detected were not listed. SFA: C12:0, C14:0, C16:0, C18:0, C20:0, C22:0. MUFA: C14:1n-9, C16:1n-7, C18:1n-7, C18:1n-9, C20:1n-9. PUFA: C18:2n-6, C18:2n-7, C18:3n-3, C20:2n-6, C20:4n-6, C20:5n-3, C22:5n-3, C22:6n-3.

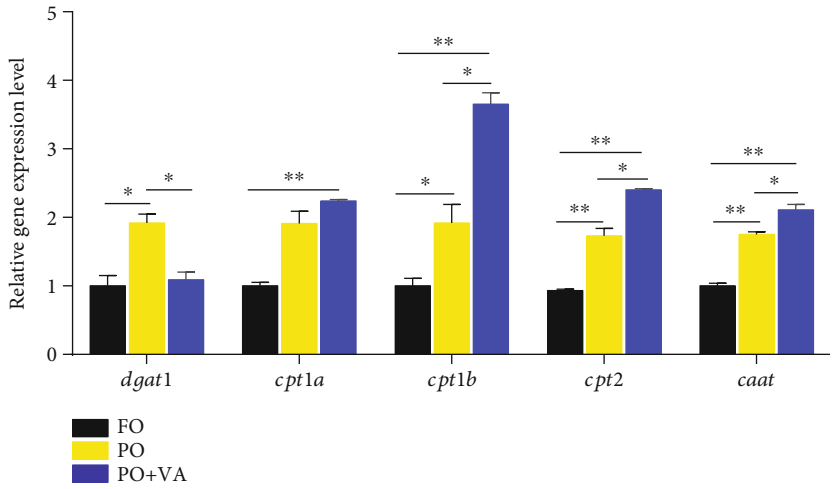


Figure 4: Gene expression related to fatty acid TG synthesis and catabolism of crabs feed different diets. Data represent means ± S.E.M. ($n = 5$). Asterisk (∗) represents a significant difference of $P < 0.05$. Double asterisks (∗∗) represent $P < 0.01$. FO (black color): fish oil; PO (red color): palm oil; PO + VA (blue color): palm oil diet with VA addition; *cpt*: carnitine palmitoyltransferase; *caat*: carnitine acetyltransferase; *dgat1*: diacylglycerol acyltransferase 1.

4. Discussion

As we know, the FO contains a balanced composition of fatty acid and rich n-3 long-chain (LC) PUFA (such as EPA and DHA), lipid-soluble substances, and attractive flavoring substances relative to PO [8]. The n-3 LC-PUFA could improve the absorption, digestion, and transport of nutrients, enhancing molting and development of crabs [7]. In addition, PO could cause a decrease the lipid digestibility and affect the growth of hybrid tilapia, *Oreochromis* sp [63]. Therefore, the best growth performance and feed utilization were found in crabs fed FO diet, keeping with the results found in swimming crabs [10]. As mentioned, VA does not exist in plants and was essential nutriment for crustacean growth [41, 47]. In the present study, VA addition in PO (PO + VA diet) relieved the growth inhibition in crabs fed PO diet (compared to those fed FO). Therefore, it was speculated that accessible vitamin A sources from FO relative to PO might also cause the discrepancy in the growth performance of crabs in the present work.

Serum vitamin A content could be raised by dietary VA and is usually used to reflect the status of dietary VA

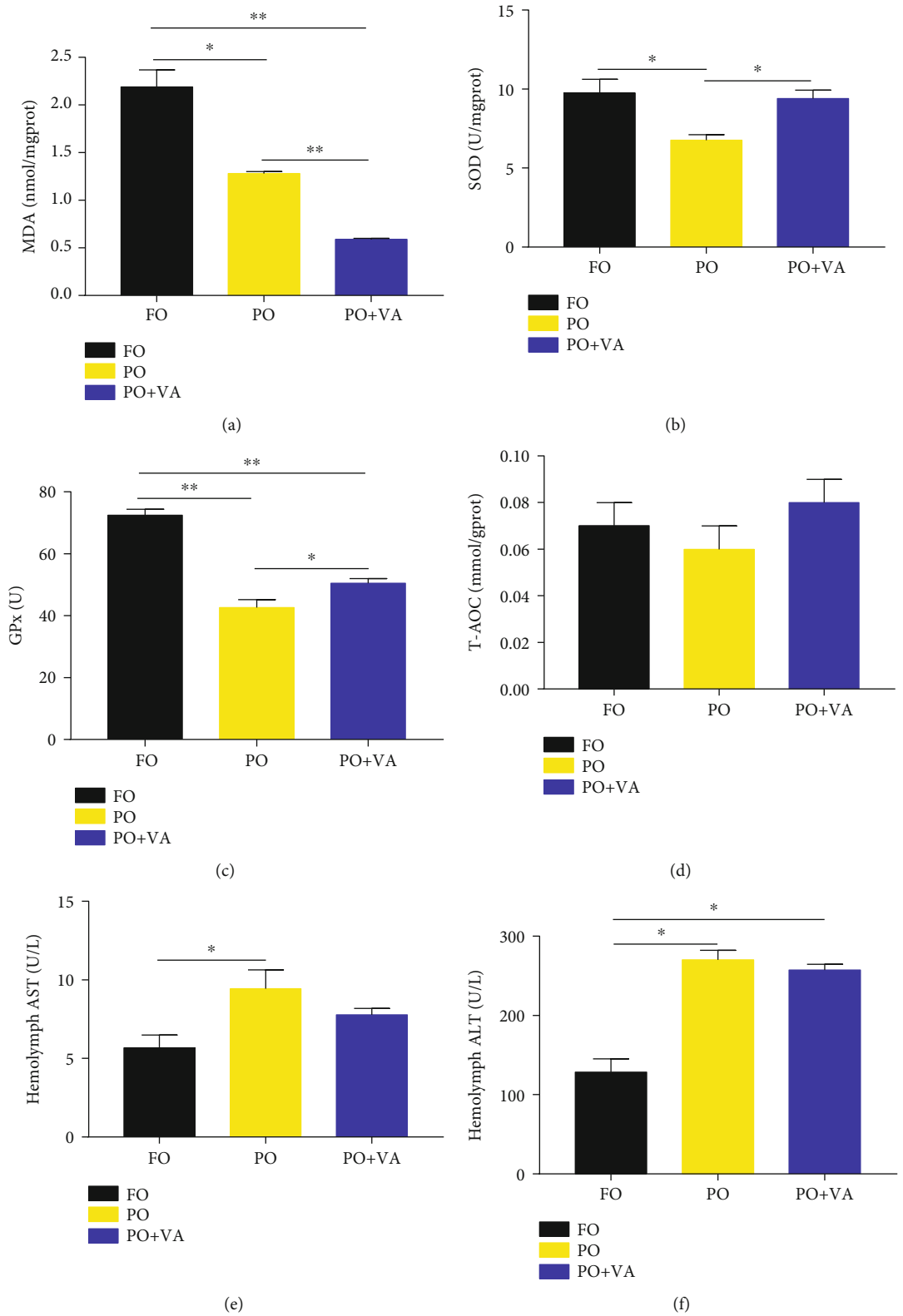


Figure 5: Antioxidant ability and health of hepatopancreas in crabs feed different diets. Data represent means ± S.E.M. ($n = 5$). Asterisk (∗) represents a significant difference of $P < 0.05$. Double asterisks (∗∗) represent $P < 0.01$. T-AOC: total antioxidation capability; SOD: superoxide dismutase; GPx: glutathione peroxidase activities; MDA: malondialdehyde content; AST: aspartate aminotransferase; ALT: alanine aminotransferase; FO (black color): fish oil; PO (red color): palm oil; PO + VA (blue color): palm oil diet with vitamin A addition.

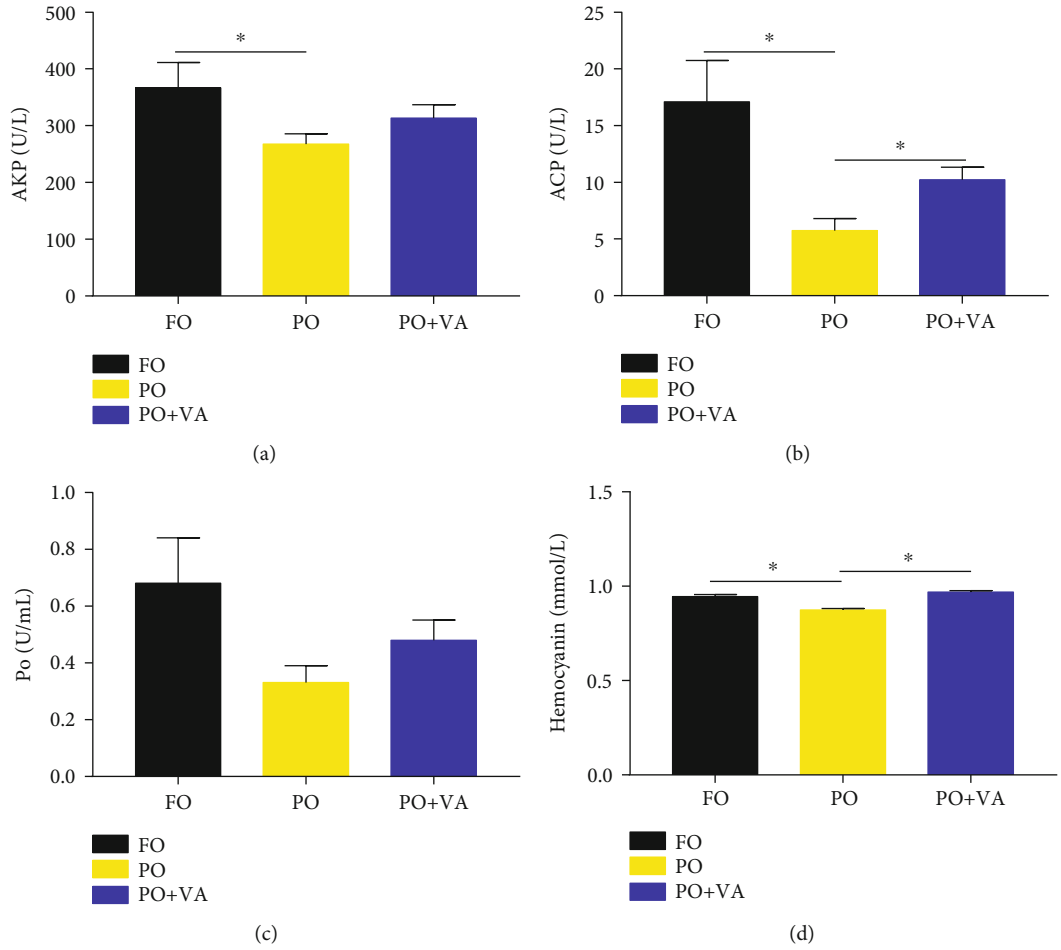


Figure 6: Immune parameters in the hemolymph of crabs feed different diets. Data represent means ± S.E.M. ($n = 5$). Asterisk (∗) represents a significant difference of $P < 0.05$. Double asterisks (∗∗) represent $P < 0.01$. AKP: alkaline phosphatase; ACP: acid phosphatase; Po: phenoloxidase; FO (black color): fish oil; PO (red color): palm oil; PO + VA (blue color): palm oil diet with vitamin A addition.

supplementation in aquatic animals [64]. It was comprehensible that VA supplementation in the PO diet increased the hemolymph VA concentration of crabs relative to those only fed PO. Whereas we found crabs fed PO diet still showed some hemolymph VA content. This might be because the crabs might have the ability to convert β-carotene, which is rich in vegetable feed, to VA. Since the key gene β-carotene 15,15′-dioxygenase has been reported in *Drosophila* [65–67], which is a typical example of an arthropod. Whereas the better growth performance of crabs fed PO + VA diets relative to those fed PO diet indicated that synthesized VA from the PO did not meet crabs' requirement, and extra VA supplementation to the PO was necessary. The reason for the growth-promoting action of dietary VA could be that it optimized lipid utilization [41, 42, 64].

The PO contains the highest level of OA, which is not easy to be utilized and is usually deposited in tissue for aquatic animals [68]. Previous studies have indicated that vegetable oils containing high OA and LA levels could induce excess lipid deposition [17, 69, 70]. Because high OA deposition in hepatopancreas could activate the gene expression of sterol regulatory element-binding protein 1 (*srebp1*), the transcription factor of lipogenesis [71]. Besides, the abundant n-3 PUFA content in the FO diet could repress the gene expression and proteolytic processing of *srebp1* [17, 72, 73]. *Dgat1* is a key rate-limiting enzyme of TG synthesis and is primarily responsible for forming lipid droplets activated by *srebp1* [74–76]. Dietary PO increased the expression of *dgat1* gene relative to the FO diet, and TG deposition in hepatopancreas was corresponsively increased in the PO group in red swamp crayfish *Procambarus clarkia* [77], in keeping with the present results. Thus, crabs fed a PO diet showed higher OA, LA, HSI, TG, and lipid content in hepatopancreas in the present study. Fatty acid β-oxidation belongs to lipid catabolism, produces energy for organisms, and requires a series of important genes, including *cpt1*, *cpt2*, and *caat* [78, 79]. SFA was the primary substrate for β-oxidation, followed by MUFA and PUFA [77]. It was shown that the PO diet caused obvious higher MUFA content in the hepatopancreas than the FO diet. Thus, the higher expression of the genes involved in fatty acids β-oxidation in crabs fed PO might be caused by the high MUFA

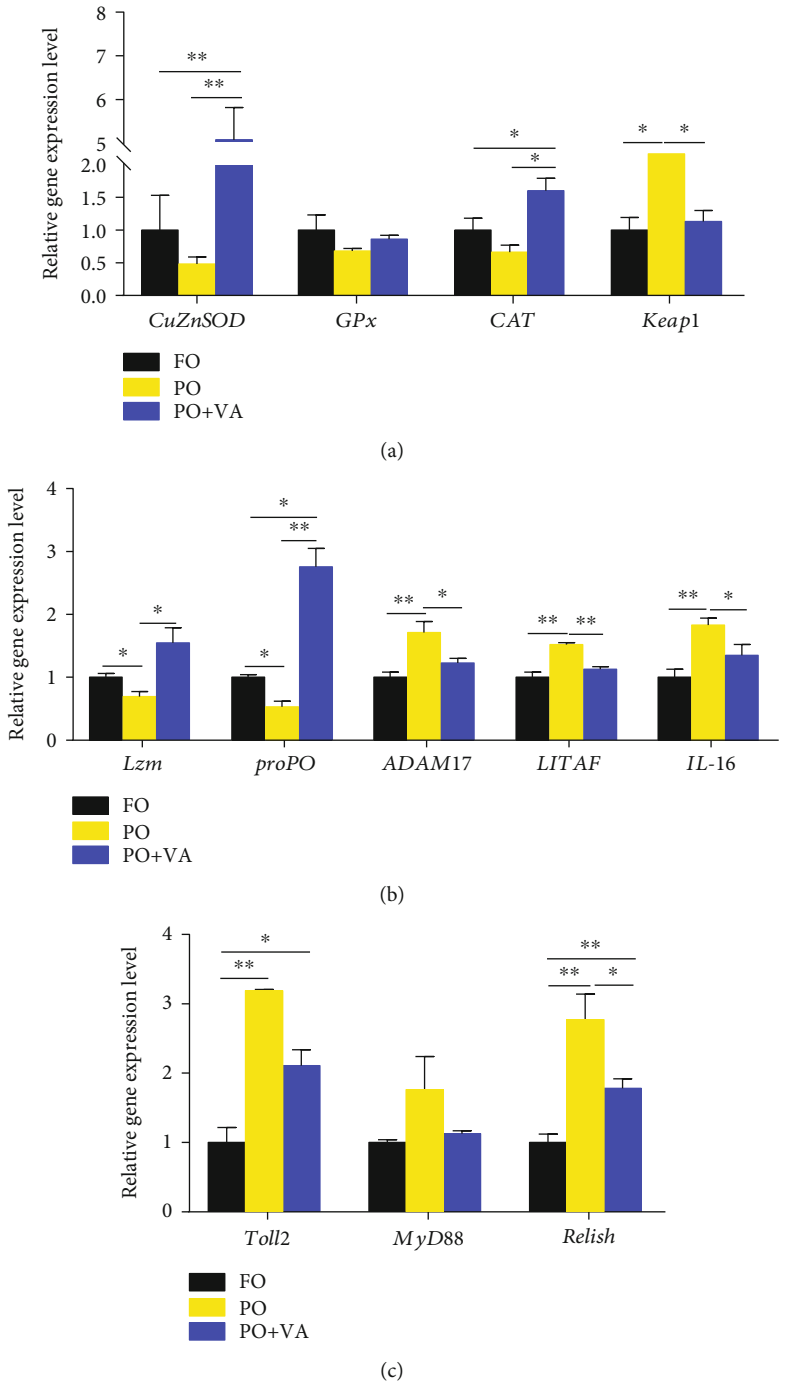


Figure 7: Gene expression of crabs related to antioxidant (a), immunity, and inflammation (b, c) feed different diets. Data represent means ± S.E.M. ($n = 5$). Asterisk (∗) represents a significant difference of $P < 0.05$. Double asterisks (∗∗) represent $P < 0.01$. FO (black color): fish oil; PO (red color): palm oil; PO + VA (blue color): palm oil diet with VA addition; *GPx*: glutathione peroxidase; *CAT*: catalase; *CuZnSOD*: Cu/Zn-superoxide dismutase; *Keap1*: Kelch-like ECH-associated protein1; *Lzm*: lysozyme; *proPO*: prophenoloxdiase; *ADAM17*: a disintegrin and metalloproteinase 17; *LITAF*: lipopolysaccharide-induced *TNFα* factor; *IL-16*: interleukin 16; *MyD88*: Myeloid differentiation factor 88.

content in the PO diet. In the meantime, high n-3 LC-PUFA content in FO also suppressed the fatty acid β-oxidation by inhibiting relevant transcription factor-peroxisome proliferator-activated receptor α (*PPARα*) [72]. The metabolite of VA, retinoic acid, might activate p38 mitogen-activated protein kinase (*p38MAPK*) and adenosine monophosphate-activated protein kinase (*AMPK*) [44], which could restrain lipid synthesis and improve fatty acid β-oxidation. Therefore, VA supplementation restrained the TG synthesis and led to a decreased tendency of lipid deposition and increased fatty acid β-oxidation in crabs in this study. Moreover, dietary VA decreased the content of

C16:0 and OA and accordingly increased LNA, DPA, and Σn-3 PUFA accumulation in hepatopancreas relative to PO. This may be because VA addition might selectively improve the utilization of different types of fatty acids. However, further investigation was needed to clarify this hypothesis. Therefore, appropriate VA addition could relieve excess lipid deposition caused by high vegetable oil levels and improve PO utilization. The improvement of energy utilization in PO by VA addition was likely to maintain normal physiology such as oxidation resistance and immunity for better growth, which are all energy-consuming processes [80–82].

It has been reported that vegetable oils containing less n-3 LC-PUFA would inactivate the nuclear factor erythroid 2-related factor 2 Nrf2 signaling pathway, weakening antioxidant activity [83, 84]. The activation of (*Nrf2*)-Kelch-like ECH-associated protein1 (*Keap1*) signaling pathway is responsible for the transcription of antioxidative genes [85]. As a result, the crabs fed PO had lower activities (T-AOC, SOD, and GPx) and gene expressions of antioxidant enzymes than those fed FO. In contrast, VA addition relieved the negative effect on antioxidant activities caused by less HUFA in the PO group. *Keap1* controls the subcellular localization and restrains the activity of *Nrf2*, and the gene expression of *Keap1* was negatively related to *Nrf2* [85–87]. Thus, the uptrend of antioxidant enzymes led by VA addition in the PO might be because the downregulated *Keap1* in the present study (the reason remained further research) released the suppression to *Nrf2* (migrating into the cell nucleus), activating the gene encoding antioxidant enzymes and leading less product of lipid peroxidation marker-MDA [86]. The antioxidant-activating role of VA was also demonstrated in grass carp, *Ctenopharyngodon Idella* [88]. Noteworthily, crabs fed FO diet caused more content of MDA in hepatopancreas relative to those fed PO, consistent with results in swimming crab [10]. It has been well acknowledged that oils containing abundant PUFA, especially EPA and DHA, were easier attacked by ROS and thus produced more product of lipid peroxidation-MDA [70]. Our results indicate that VA addition might improve hepatopancreas health by enhancing the antioxidant ability.

Whereas attenuation of antioxidant ability usually impaired immunity in crustaceans [89]. Crabs depend on the innate immune system due to a lack of an acquired immune system [90]. The hemocyanin, a susceptive and multifunctional protein, and the hydrolytic lysosomal enzymes such as AKP, ACP, and LZM are involved in the humoral immune responses of fishes and crustaceans [58, 91, 92]. The proPO system, only found in invertebrates, could activate the Po when faced with pathogens invasion [90]. The decreases in enzyme activities (hemocyanin, Po, AKP, and ACP) and expression of partial immune genes (*LZM* and *proPO*) indicated an attenuation of immunity caused by the PO diet in the present study. Cytokines are also an essential part of innate immunity for aquatic animal [93]. *IL-16* plays an essential part in initiating innate immune responses and promoting a proinflammatory response [94]. *LITAF*, an important transcription factor, activates the transcription of *TNFα* and other cytokines and regulates inflammatory response [95]. *NF-κB* was reported to be a key regulator of *ADAM17* action (a *TNFα*-converting enzyme) [96, 97], and the activation of *NF-κB* would promote the expression of proinflammatory factors such as *IL-16* and *TNFα* [30, 98]. *Toll2* was a member of pattern recognition receptors (PRRs), which could interact with the adaptor molecule *MyD88* and trigger downstream signaling events, activating *NF-κB* and influencing inflammatory cytokine production [99, 100]. Thus, the PO diet might induce the expression of *IL-16*, *ADAM17*, and *LITAF* and weaken immunity through activating *Toll2*/*MyD88*/*Relish* (*NF-κB*-like transcription factor), according to the current results. It has been reported that n-3 PUFA in the FO has a role in inflammation resistance [29, 101, 102]. The high content of LA and OA in PO oil could induce the transcription of pro-inflammatory factors [103, 104]. The difference in fatty acid composition might be one reason for the anabatic inflammatory response in the PO diet. However, VA addition to the PO diet showed an anti-inflammatory action and relieved proinflammatory response caused by PO. In addition, the activation of the *Nrf2-Keap1* signaling pathway affected by VA could weaken the proinflammatory cytokine production [81, 101]. Similarly, VA's anti-inflammatory and immunity-stimulating role has also been reported in hybrid grouper, *Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂ [105] and grass carp, *Ctenopharyngodon Idella* [88]. The attenuation of proinflammatory response corresponds to the enhanced antioxidant system by VA supplementation.

5. Conclusion

In conclusion, total replacement of FO by PO caused abnormal lipid metabolism and excess lipid accumulation in the crab's hepatopancreas and impaired antioxidant ability and immunity in the hepatopancreas. At the same time, VA supplementation could improve the utilization of fatty acids (C12:0, C16:0, and OA) and antioxidant-related gene expression in hepatopancreas. VA also relieved the inflammation response and improved the immunity, possibly through *Toll2*/*MyD88*/*Relish* signaling pathway. These results indicated the protective role of VA on hepatopancreas health. This study conveyed the importance of VA addition in natural PO. Furthermore, it offered novel insights into the application of VA as a feed additive in crabs fed dietary FO replacement by vegetable oil.

Data Availability

All data are available upon request by contact with the corresponding author.

Ethical Approval

This research followed the Guidance of the Care and Use of Laboratory Animals in China, and the protocol was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (no. f20201001).

Conflicts of Interest

No competing interest exists in this paper.

Authors' Contributions

Qincheng Huang conducted the farmed experiment, analyzed the data, and wrote the manuscript. Xiaodan Wang modified the manuscript and offered suggestions for the experiment. Jiadai Liu, Han Wang, Yixin Miao, and Cong Zhang helped with farmed experiment and sampling. Meiling Zhang and Chuanjie Qin offered suggestions for the experiment. Jianguang Qin improved data interpretation, review of the manuscript, and language usage. Liqiao Chen helped design the experiment, review the manuscript, and subsidize funding.

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