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High percentage of dietary palm oil suppressed growth and antioxidant capacity and induced the inflammation by activation of TLR-NF-κB signaling pathway in large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

A 70-day feeding trial was conducted to investigate the effects of dietary fish oil (FO) replaced by palm oil (PO) on growth, biochemical and antioxidant response as well as inflammatory response in the liver of large yellow croaker (initial weight 15.87 ± 0.14 g). Four iso-proteic and iso-lipidic experimental diets were formulated with 0% (the control group), 33.3%, 66.7% and 100% FO replaced by PO. Fish fed the diet with 100% PO showed significantly lower growth performance than the control group. As expected, the contents of C16:0, C18:1n-9 and C18:2n-6 were increased with increasing dietary PO levels. There were remarkable increases in total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) levels in fish fed the diet with 100% PO compared to the control group. Moreover, dietary PO significantly increased activities of plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) in fish fed the diet with 100% PO compared to the control group. The total antioxidant capacity (T-AOC) and the activity of catalase (CAT) in plasma were significantly decreased in fish fed the diet with 100% PO compared to the control group, and meanwhile no significant differences were found in T-AOC and CAT activity in fish fed diets with no more than 66.7% PO. Fish fed the diet with 100% PO exerted significantly higher toll like receptors (TLRs) and myeloid differentiation factor (MyD88) mRNA expression levels than the control group. The IFNγ, IL-1β and TNFα mRNA expressions were increased with increasing dietary PO levels. The increase of pro-inflammatory gene expression may be due to the activation of NF-κB signaling as the ratio of nucleus p65 to total p65 protein was elevated with the increase of dietary PO levels. These results showed that relatively higher PO levels in diets suppressed the growth and antioxidant capacity as well as induced the inflammatory response by activating TLR-NF-κB signaling pathway in juvenile large yellow croaker.

1. Introduction

With the increasing demand of fish oil (FO) and limited supply of fishery resources, researchers have been making progress towards decreasing the use of FO with increasing use of vegetable oil in aquafeeds. Palm oil (PO) is the second most highly produced seed oil, and it is regarded as a promising alternative to FO in fish feed [1]. Studies have shown that FO can be partially replaced by PO without significantly affecting the growth performance of African catfish (*Clarias gariepinus*) [2], red hybrid tilapia (*Oreochromis* sp.) [3] and gilthead sea bream (*Sparus aurata*) [4]. These investigations have been carried out on the effects of growth [5], tissue fatty acid profiles [2], biochemical and antioxidant responses [6–8] when PO is used as a lipid source in aquafeed. However, little information is available on the effects of dietary PO on inflammatory response in fish species, although some studies have been conducted in the mammals [9–11].

PO is rich in saturated fatty acid (SFA), palmitic acid. Studies in mammals have demonstrated that consumption of SFA strongly regulates the immune function and inflammatory signaling in adipose tissue [12]. SFA has been reported to stimulate toll-like receptor 4

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(TLR4) signaling to induce IL-6 and TNFα expression in macrophages [13]. Nuclear factor-κB (NF-κB), a vital regulator of inflammatory pathways, plays an important role in the inflammatory response induced by SFA [14,15]. The NF-κB/Rel family includes p50/p105, p65/RelA, c-Rel, Rel B, and p52/p100 subunits. Most members of this family can form heterodimers with each other, excluding Rel B that can homodimerize. The most prevalent activated form of NF-κB is a heterodimer consisting of p65 with p50 or p52 subunit, which contains transactivation domains that are necessary for gene induction [16,17]. Therefore, the p65 subunit is regarded as the biomarker protein of the NF-κB transcription factor. Furthermore, inflammation in fish has also been an attractive field for scientists as it is important to understand its immunomodulatory mechanism and maintain fish health [18]. However, till today, there has not been any study which would provide detailed information about the role of PO, enriched with SFA, on NF-κB activation and inflammatory response in fish.

Large yellow croaker (*Larimichthys crocea*), with the highest yield among mariculture fish in China, has been widely cultured in the southeast of China [19]. The use of PO in large yellow croaker diet has a promising prospect. However, it is still not clear what are the effects on antioxidant response and inflammatory response when FO is replace by PO in this species. The overall objective of this study was to identify the effects of dietary FO replaced by PO on growth performance, fatty acid profiles, antioxidant response and TLR-NF-κB signaling in large yellow croaker for the utmost usage of PO in aquafeed.

2. Materials and methods

2.1. Experimental diets

Four iso-nitrogenous (42% protein content) and iso-lipidic (12% lipids content) diets were formulated with 0% (the control group), 33.3%, 66.7% and 100% FO replaced by PO (Table 1). All raw materials of feed were crushed through 75 μm mesh. Ingredients were mixed thoroughly and the mixture was used to make pellets of 4.0 × 4.0 mm and 4.0 × 6.0 mm sizes by an automatic pellet-making machine (F-26 (II), South China University of Technology, China). The pellets were dried for 10 h at 45 °C in oven. After drying, the pellets were stored in a refrigerator at −20 °C until use and analysis. Fatty acid profiles of all four diets were determined (Table 2).

Table 1
Formulation and chemical proximate analysis of the experimental diets (% dry weight).

Ingredients[a]	Palm oil replacement level/%			
	0% (the control)	33.3%	66.7%	100%
White fish meal[b]	35	35	35	35
Soybean meal[b]	28	28	28	28
Wheat meal[b]	23.8	23.8	23.8	23.8
Soybean lecithin	1.5	1.5	1.5	1.5
Vitamin premix[c]	2	2	2	2
Mineral premix[c]	2	2	2	2
Attractant mixture[d]	0.1	0.1	0.1	0.1
Mould inhibitor[e]	0.1	0.1	0.1	0.1
Fish oil	7.5	5.0	2.5	0
Palm oil	0	2.5	5.0	7.5
Total	100	100	100	100
Proximate analysis (dry matter %)				
Crude protein	42.23	42.07	41.97	42.37
Crude lipid	12.05	12.31	12.62	12.53

[a] All ingredients purchased from Great Seven Biotechnology Co., Ltd, China.
[b] Fish meal (dry mater, %): 70.55% crude protein and 7.21% crude lipid; Soybean meal (dry mater, %): 51.89% crude protein and 1.16% crude lipid; Wheat meal (dry mater, %): 15.09% crude protein and 0.15% crude lipid.
[c] The mixture of mineral mixture and vitamin mixture according to Yan et al. [51].
[d] Attractant: the mixture of 50% glycine acid and 50% betaine by weight.
[e] Mold inhibitor: the mixture of 50% calcium propionic acid and 50% fumaric acid by weight.

Table 2
Fatty acid profiles of palm oil and the experimental diets (% total fatty acids)[f].

Fatty acid (% total fatty acids)	Palm oil	Palm oil replacement level/%			
		0% (the control)	33.3%	66.7%	100%
14:0	0.92	6.57	4.47	2.82	1.25
16:0	43.01	22.79	28.32	34.15	39.51
18:0	3.19	5.58	5.06	4.74	4.29
20:0	0.24	1.45	1.63	1.42	1.29
ΣSFA[a]	47.36	36.40	39.47	43.14	46.35
16:1n-7	0.25	8.72	5.93	3.93	1.28
18:1n-9	30.12	13.59	17.59	21.58	25.61
ΣMUFA[b]	30.37	22.30	23.52	25.50	26.89
18:2n-6	12.10	11.26	12.87	14.43	15.96
20:4n-6	0.00	1.12	0.75	0.52	0.31
Σn-6PUFA[c]	12.10	12.38	13.62	14.95	16.27
18:3n-3	0.74	1.50	1.29	1.36	1.30
20:5n-3	0.00	7.06	4.73	2.79	1.06
22:6n-3	0.00	5.38	3.80	2.60	1.49
Σn-3PUFA[d]	0.74	13.94	9.81	6.74	3.84
n-3/n-6PUFA	0.06	1.13	0.72	0.45	0.24
Σn-3LC-PUFA[e]	0.00	12.44	8.53	5.38	2.54

[a] SFA: saturated fatty acids.
[b] MUFA: mono-unsaturated fatty acids.
[c] n-6 PUFA: n-6 poly-unsaturated fatty acids.
[d] n-3 PUFA: n-3 poly-unsaturated fatty acids.
[e] LC-PUFA: long chain-polyunsaturated fatty acids.
[f] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C20:0, C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C18:3n-6, C20:3n-6, C18:4n-3, C22:5n-3, are not listed in the table.

The method of analyzing fatty acid profiles was based on the procedure described by Metcalfe, Schmitz and Pelka (1966) [20] with some modifications [21,22]. Fatty acids were prepared by saponification of total lipids in freeze-dried samples (100.0 mg) with 3 ml KOH–ethanol (1 N), and acid-catalyzed methylation with 3 ml methanolic hydrogen chloride (2 N). After extraction and purification of fatty acid methyl esters (FAME) as described by Zuo et al. (2012) [21], FAME were quantified by a HP6890 gas chromatograph (Agilent Technologies Inc., Santa Clara, California, USA) with a fused silica capillary column (007-CW, Hewlett Packard, Palo Alto, CA, USA). The column temperature was programmed to rise from 150 °C up to 200 °C at a rate of 15 °C min^{-1}, and from 200 °C to 250 °C at a rate of 2 °C min^{-1}. Both injector and detector temperatures were 250 °C. Results are presented as a percentage of total fatty acids.

2.2. Fish culture and sample collection

Disease-free, homogenous size of large yellow croakers were supplied by Ningde Fufa Fishery co., Ltd., Fujian, China. Before the actual feeding trial, 1500 fish were acclimated to the control diet and the experimental environment in floating sea cages (2 × 4 × 2 m) for 14 days. 720 fish (average weight 15.87 ± 0.14 g) were used in this experiment, and the juvenile of 60 fish per cage were stocked into triplicate floating cages (1 × 1 × 1.8 m) in seawater. Fish were hand-fed to apparent satiation twice daily (05:00 and 17:00) for 70 days.

After the feeding experiment, large yellow croaker were starved for 24 h and anesthetized with MS222 (1:10 000; Sigma, USA) before all fish were counted and weighed for measuring the survival rate, condition factor and specific growth rate. Five fish from each cage were randomly sampled for the analysis of the body composition. Blood samples were obtained from six fish per cage and clotted at room temperature for 4 h. After then, the blood was centrifuged to collect plasma. The plasma was stored in −80 °C refrigerator for future

analysis. The liver and visceral of six fish from each cage were weighed for measuring the hepatosomatic index (HSI) and viscerosomatic index (VSI). The liver of twelve fish from each cage were randomly sampled and stored into 1.5 ml Eppendorf tubes. The tubes were frozen into liquid nitrogen, and then stored at −80 °C for antioxidant capacity and gene expression analysis. The liver and muscle of another six fish from each cage were pooled into 5 ml tubes, frozen into liquid nitrogen and then stored at −80 °C for the assay of fatty acid composition, moisture and crude lipids.

The present study was performed in strict accordance with the Standard Operation Procedures (SOPs) of the Institutional Animal Care and Use Committee of the Ocean University of China.

2.3. Biochemical and antioxidant capacity analysis

Crude lipid, crude protein and moisture contents of the diets and fish were analyzed according to the procedures of the Association of Official Analytical Chemists (AOAC, 1995) [23]. The lipid contents of liver and muscle were analyzed using chloroform-methanol method [24,25]. The contents of triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) in plasma, as well as activities of serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured using BS180 automatic biochemical analyzer (BS-400; Mindray, China) with the matching commercial reagents and kits. The content of malondialdehyde (MDA), and the activities of superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and catalase (CAT) in the liver were determined using commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. RNA extraction, cDNA synthesis and real-time quantitative polymerase chain reaction

The liver was pulverized in liquid nitrogen and Trizol reagent (Takara, Japan) was added to extract the total RNA following the manufacturer's protocol. The total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water, and the quality of RNA was evaluated by a 1.2% denaturing agarose gel and the total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA was treated with RNase-Free DNase (Takara, Japan) to remove DNA contaminant and was reversely transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Japan) as per the manufacture's instructions.

Primers (Table 3) for the real-time quantitative polymerase chain reaction (RT-qPCR) were designed by the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, Canada) based on the nucleotide sequences or referred to Tan et al. [26]. The β-actin was considered as the house keeping gene in this study. The amplification was performed in a total volume of 25 μL containing 1 μL of each primer, 1 μL of cDNA, 12.5 μL of SYBR® Premix Ex Taq™ II (Takara, Japan), 9.5 μL of RNase-free water. The RT-qPCR program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 20 s. Melting curve analysis was carried to confirm that a single PCR product was present in these reactions at the end of the PCR reaction. Standard curves were prepared with 4-fold serial dilutions (in triplicate) of cDNA that was used to calculate the amplification efficiency using the following equation: $E = 10^{(-1/slope)} - 1$. The amplification efficiencies of the target and reference genes primers ranged between 0.95 and 1.05. The gene expression levels were calculated with the $2^{-\Delta\Delta CT}$ method [27].

2.5. Western blot analysis

Total liver tissue protein was extracted with Glass Tenbroeck Tissue Grinders (Kimble Chase) according to previously described methods [28]. The nuclear protein of liver tissue was extracted by using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Protein concentration was determined by a BCA Protein Assay Kit (Beyotime Biotechnology, China). Equal amounts of protein sample (20 μg) were loaded into wells and separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were transferred to activated polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Further, the PVDF membranes containing the proteins were blocked by 5% skimmed milk powder dissolved in tris buffered saline with Tween™ (TBST) at room temperature for 2 h. After washed with TBST five times for 3 min each time, the membranes were incubated with primary antibody dissolved in TBST for overnight in the 4 °C freezer. Next day the membranes were washed with TBST for five times, 3 min each time and then incubated for 2 h with secondary antibody dissolved in TBST. Immune complexes were visualized by using a Beyo ECL Plus kit (Beyotime Biotechnology, China). The density of protein bands was quantified by the ImageJ 1.48 software (National Institutes of Health, USA). The total p65 (t-p65) was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and nucleus p65 (n-p65) was normalized against Histone H3.

Polyclonal anti-p65 antibody was purchased from Cell Signaling Technology (USA). Anti-Histone H3 antibody was obtained from Abcam

Table 3
Sequences of the PCR primers used in this study.

Target gene	Forward primers (5′–3′)	Reverse primers (5′–3′)	Accession number
Nrf2	GATGGAAATGGAGGTGATGC	CATGTTCTTTCTGTCGGTGG	XM 010737768
SOD1	GCGGGACCGTGTTCTTTGAG	GCTACCAGCGTTGCCAGTCTTT	NM 001303360
SOD2	GGCACTGGCAAAGGGAGAC	CACAAGCGGCGATACGAAG	NM 001303364
CAT	CTTCATCAGGGACGCTCTACT	ATGGCATAATCTGGGTTGGT	XM 010735178
TLR2	TCTGCTGGTGTCAGAGGTCA	GGTGAATCCGCCATAGGA	KJ820743
TLR3	ACTTAGCCCGTTTGTGGAAG	CCAGGCTTAGTTCACGGAGG	HQ589262
TLR9	AACGGAGGTCACAGGAGG	TAGCACCACTGGACAGCAC	EU655705
TLR13	CCTCCTGTTTATGGTAGTGTCC	GCTCGTCATGGGTGTTGTAG	NM 001303396
TLR22	TATGCGAGCAGGAAGACC	CAGAAACACCAGGATCAGC	GU324977
MyD88	TACGAAGCGACCAATAACCC	ATCAATCAAAGGCCGAAGAT	EU978950
IL-6	CGACACACCCACTATTTACAAC	TCCCATTTTCTGAACTGCCTCT	KU140675
IFNγ	TCAGACCTCCGCACCATCA	GCAACCATTGTAACGCCACTTA	KM501500
IL-1β	CATAGGGATGGGGACAACGA	AGGGGACGGACACAAGGGTA	KJ459927
TNFα	ACACCTCTCAGCCACAGGAT	CCGTGTCCCACTCCATAGTT	EF165623
IL-10	AGTCGGTTACTTTCTGTGGTG	TGTATGACGCAATATGGTCTG	XM010738826
Arg1	AACCACCCGCAGGATTACG	AAACTCACTGGCATCACCTCA	XM019269015
β-actin	GACCTGACAGACTACCTCATG	AGTTGAAGGTGGTCTCGTGGA	GU584189

Nrf2: nuclear factor erythroid 2-related factor 2, SOD: superoxide dismutase, CAT: catalase, TLR: toll-like receptor, MyD88: myeloid differentiation factor 88, IL-6: interleukin-6, IFNγ: interferon γ, IL-1β: interleukin-1β, TNFα: tumour necrosis factor α, IL-10: interleukin-10, Arg-1: arginase-1.

(England). Anti-GAPDH and HRP-conjugated secondary antibodies were obtained from Golden Bridge Biotechnology (China).

2.6. Calculations and statistical analysis

Survival rate (SR%) = Nt × 100 / N_0

Specific growth rate (SGR, % day^{-1}) = (Ln Wt - Ln W_0) × 100 / t

Feed efficiency ratio (FER) = wet weight gain in g / dry feed fed in g

Feed intake (FI,% day^{-1}) = dry feed intake in g × 100 / [(W0 + Wt) / 2] / t

Feed conversion ratio (FCR) = dry feed fed in g / wet weight gain in g

Hepatosomatic index (HSI%) = liver wet weight × 100 / body wet weight

Viscerosomatic index (VSI%) = visceral wet weight × 100 / body wet weight

Condition factor (CF%) = 100 × Wt / (Lt^3)

Where Nt and N_0 were final and initial fish number, respectively; Wt and W_0 were final and initial fish weight, respectively; t was period of experimental days; Lt was final body length.

All statistics were evaluated by SPSS 19.0 (IBM, America). All data were subjected to a one-way analysis of variance (ANOVA) and followed by Tukey's multiple-range test. The level of significance was chosen at $P < 0.05$ and the results were presented as means ± S.E.M. (standard error of the mean).

3. Results

3.1. Survival, growth performance and body index

At the end of the feeding experiment, no significant differences were observed in SR and FI of fish fed grade levels of PO ($P > 0.05$). The specific growth rate significantly decreased from 1.24% day^{-1} to 0.99% day^{-1} with the increase of dietary PO levels. There was no significant difference in the specific growth rate among fish fed diets with 0% (the control group), 33.3% and 66.7% PO. However, fish fed the diet with 100% PO showed significantly lower growth rate than the control group. Similar results were obtained in FER, where remarkable decrease was noticed in fish fed the diet with 100% PO than the control group ($P < 0.05$). FCR significantly increased from 1.74 to 2.41 with the increase of dietary PO levels. No significant differences were found in HSI, VSI and CF of fish fed diets with graded levels of PO (Table 4).

3.2. Body composition analysis

Body composition analysis showed that the lipid content of whole body increased from 7.31% to 8.74%, which was significantly higher in fish fed the diet with 100% PO compared to the control group ($P < 0.05$). However, no significant differences were observed in the protein and moisture contents of whole body among dietary treatments. With increasing dietary PO, the lipid content of the liver significantly increased from 23.09% to 31.23% ($P < 0.05$). When fish were fed the diet with 100% PO, the lipid content of the liver was significantly higher than the control group ($P < 0.05$). There were no significant differences in the moisture content of liver, the moisture and lipid contents of muscle in fish fed diets with graded levels of PO (Table 5).

3.3. Fatty acid profiles in the liver of large yellow croaker

Significant increases were observed in the contents of C16:0, C18:1n-9 and C18:2n-6 fatty acid in the liver of fish fed the diet with 100% PO compared to the control group. On the contrary, the proportion of hepatic EPA and DHA, two kinds of n-3 LC-PUFA, decreased markedly in fish fed the diet with 66.7% PO than the control group. Further, compared to the control group, the content of C20:4n-6 was significantly decreased in the liver of large yellow croaker fed the diet with 100% PO ($P < 0.05$). The maximum contents of n-6 PUFA (13.09%) and total SFA (40.15%) were detected in fish fed the diet with 100% PO among dietary treatments. The ratio of n-3 PUFA to n-6 PUFA in fish fed the diet with 100% PO showed a significant decrease compared to the control group ($P < 0.05$), but no significant difference was found in the content of MUFA in fish among dietary treatments (Table 6) ($P > 0.05$).

3.4. Plasma biochemical indexes

As dietary PO levels increased, significantly increasing trends were observed in the contents of LDL-C (from 0.71 to 1.17 mmol/L) and TC (from 2.69 to 3.61 mmol/L) ($P < 0.05$). However, the content of HDL-C in plasma was decreased from 0.77 mmol/L in fish of the control group to 0.50 mmol/L in fish fed the diet with 100% PO. When PO level was more than 66.7%, the content of HDL-C in plasma was significantly lower than the control group ($P < 0.05$). No significant difference was detected in the content of TG in plasma among dietary treatments ($P > 0.05$). Activity of serum AST increased significantly in fish fed the diet with 100% PO than the control group. Moreover, compared to the control group, activity of serum ALT showed a significant increase in fish fed the diet with 66.7% PO (Table 7).

3.5. Antioxidant enzyme activities and related gene expression

Concentration of hepatic MDA (Fig. 1a.) of large yellow croaker fed the diet with 100% PO was significantly increased than the control

Table 4
Growth, survival and somatic parameters of large yellow croaker (*Larmichthys crocea*) fed diets with graded levels of fish oil replaced by palm oil (Means ± S.E.M)[a].

Index	0% (the control)	33.3%	66.7%	100%
Initial body weight (IBW, g)	15.88 ± 0.36	15.86 ± 0.26	15.88 ± 0.29	15.87 ± 0.36
Final body weight (FBW, g)	37.76 ± 0.23[a]	36.69 ± 0.81[a]	35.47 ± 1.97[ab]	31.64 ± 0.24[b]
Survival rate (SR, %)	91.11 ± 0.56	87.78 ± 0.56	89.44 ± 2.42	87.78 ± 1.47
Specific growth rate (SGR, % day^{-1})	1.24 ± 0.02[a]	1.20 ± 0.01[ab]	1.14 ± 0.10[ab]	0.99 ± 0.03[b]
Feed Intake (FI, %/d)	2.03 ± 0.05	2.10 ± 0.07	2.20 ± 0.07	2.28 ± 0.01
Feed efficiency ratio (FER)	0.57 ± 0.01[a]	0.54 ± 0.02[ab]	0.50 ± 0.05[ab]	0.42 ± 0.01[b]
Feed conversion ratio (FCR)	1.74 ± 0.04[b]	1.85 ± 0.08[b]	2.06 ± 0.21[ab]	2.41 ± 0.06[a]
Hepato-somatic index (HSI, %)	1.90 ± 0.09	1.98 ± 0.07	2.19 ± 0.08	2.08 ± 0.11
Viscera-somatic index (VSI, %)	7.08 ± 0.33	6.41 ± 0.26	6.62 ± 0.17	6.25 ± 0.36
Condition Factor (CF, %)	1.02 ± 0.02	1.05 ± 0.05	1.14 ± 0.04	1.04 ± 0.04

[a] Data are presented as means ± S.E.M. Means in each row sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means.

Table 5
Body composition analysis of large yellow croaker (*Larmichthys crocea*) fed diets with graded levels of fish oil replaced by palm oil (Means ± S.E.M)[a].

Index (wet weight, %)	0% (the control)	33.3%	66.7%	100%
Whole body (%)				
Moisture	72.31 ± 0.42	73.31 ± 0.40	73.37 ± 0.28	72.25 ± 0.18
Lipid	7.31 ± 0.29[b]	7.39 ± 0.18[b]	7.90 ± 0.24[ab]	8.74 ± 0.48[a]
Protein	15.50 ± 0.31	16.13 ± 0.24	15.53 ± 0.08	15.70 ± 0.12
Liver (%)				
Moisture	59.77 ± 1.09	54.97 ± 2.72	54.74 ± 2.80	52.06 ± 0.92
Lipid	23.09 ± 1.16[b]	25.60 ± 2.59[ab]	26.68 ± 1.76[ab]	31.23 ± 0.80[a]
Muscle (%)				
Moisture	73.49 ± 1.05	74.43 ± 1.87	73.24 ± 0.98	74.25 ± 1.75
Lipid	7.86 ± 0.70	8.39 ± 1.08	7.80 ± 0.89	8.88 ± 1.09

[a] Data are presented as means ± S.E.M. Means in each row sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means.

group ($P < 0.05$). Meanwhile, no remarkable difference was detected in the activity of SOD (Fig. 1b.) among dietary treatments ($P > 0.05$). As dietary PO increased, activity of T-AOC decreased from 1.21 to 0.76 U/mgprot, and activity of CAT decreased from 15.10 to 9.27 U/mgprot (Fig. 1c and d.) ($P < 0.05$). However, no significant differences were detected in activities of T-AOC and CAT in fish fed the diet with 66.7% PO and the control group. The SOD1, CAT and nuclear factor-erythroid 2-related factor-2 (Nrf2) mRNA expression (Fig. 2.) was significantly decreased when fish were fed the diet with 100% PO compared to the control group ($P < 0.05$). No significant difference was detected in the SOD2 mRNA expression (Fig. 2.) among dietary treatments ($P > 0.05$).

3.6. The mRNA expression of TLR signaling pathway and the inflammation in large yellow croaker

The TLR2, TLR9 and TLR22 mRNA expression was significantly elevated when fish were fed the diet with 66.7% PO compared to the control group ($P < 0.05$). The TLR3 mRNA expression in fish fed the diet with 100% PO was significantly higher than the control group. Additionally, when compared to the control group, there was a significant increase of transcript level of MyD88 when fish were fed the diet with 33.3% PO ($P < 0.05$). However, there was no significant difference in TLR13 mRNA expression among fish fed each diet ($P > 0.05$) (Fig. 3a.). The IFNγ, IL-1β and TNFα mRNA expression level was the highest in large yellow croaker fed the diet with 100% PO than those were fed other diets ($P < 0.05$). IL-10 mRNA expression showed an adverse tendency of gene transcript level from 1.03 to 0.51, and the lowest mRNA level was observed in fish fed the diet with 100% PO among dietary treatments. As dietary PO increased, the expression level of Arg-1 decreased from 1.01 to 0.58, but no significant difference was observed in Arg-1 mRNA expression among all dietary treatments (Fig. 3b).

3.7. Western blot for NF-κB signaling

In order to investigate whether NF-κB participated in inflammatory response in the liver of juvenile fish fed the diet containing PO or not, nucleus p65 (n-p65), total p65 (t-p65) protein expression were detected by western blot analysis. The ratio of n-p65 to t-p65 protein increased from 0.73 to 0.99 with increasing dietary PO. The ratio of n-p65 to t-p65 protein in the liver of juvenile fish was significantly increased in fish fed the diet with 100% PO compared to the control group ($P < 0.05$). There was no significant difference in the ratio of n-p65 to t-p65 protein in fish fed the diet with 66.7% PO compared to the control

Table 6
Fatty acid profiles (% total fatty acids) in the liver of large yellow croaker (*Larmichthys crocea*) fed diets with graded levels of fish oil replaced by palm oil (Means ± S.E.M)[f].

Fatty acid (% total fatty acids)	0% (the control)	33.3%	66.7%	100%
14:0	4.66 ± 0.18[a]	2.97 ± 0.25[b]	2.39 ± 0.06[bc]	1.71 ± 0.01[c]
16:0	21.98 ± 0.45[c]	25.08 ± 0.35[b]	27.87 ± 0.15[b]	30.57 ± 0.48[a]
18:0	7.49 ± 0.68	7.00 ± 0.66	6.74 ± 0.09	6.36 ± 0.28
20:0	1.70 ± 0.13	1.43 ± 0.13	1.55 ± 0.02	1.51 ± 0.07
ΣSFA[a]	35.83 ± 1.29[b]	36.48 ± 0.48[b]	38.56 ± 0.14[ab]	40.15 ± 0.49[a]
16:1n-7	12.91 ± 0.24[a]	10.30 ± 0.38[b]	8.40 ± 0.26[c]	6.10 ± 0.18[d]
18:1n-9	21.43 ± 0.34[d]	23.65 ± 0.50[c]	26.29 ± 0.24[b]	29.24 ± 0.36[a]
ΣMUFA[b]	34.34 ± 0.59	33.95 ± 0.86	34.69 ± 0.43	35.34 ± 0.52
18:2n-6	7.65 ± 0.99[c]	8.76 ± 0.57[bc]	11.37 ± 0.48[ab]	12.96 ± 0.89[a]
20:4n-6	0.49 ± 0.07[a]	0.35 ± 0.06[ab]	0.27 ± 0.02[bc]	0.13 ± 0.02[c]
Σn-6PUFA[c]	8.14 ± 0.96[c]	9.11 ± 0.59[bc]	11.64 ± 0.49[ab]	13.09 ± 0.91[a]
18:3n-3	1.87 ± 0.19	1.53 ± 0.25	1.49 ± 0.35	1.51 ± 0.29
20:5n-3(EPA)	3.47 ± 0.36[a]	1.94 ± 0.09[b]	1.00 ± 0.16[c]	0.57 ± 0.08[c]
22:6n-3(DHA)	1.92 ± 0.28[a]	1.45 ± 0.18[ab]	0.69 ± 0.06[bc]	0.31 ± 0.04[c]
Σn-3PUFA[d]	7.26 ± 0.60[a]	4.92 ± 0.19[b]	3.17 ± 0.56[bc]	2.39 ± 0.27[c]
n-3/n-6PUFA	0.90 ± 0.08[a]	0.55 ± 0.05[b]	0.27 ± 0.04[c]	0.19 ± 0.03[c]
Σn-3LC-PUFA[e]	5.38 ± 0.55[a]	3.38 ± 0.16[b]	1.69 ± 0.22[c]	0.88 ± 0.09[c]

[a] SFA: saturated fatty acids.
[b] MUFA: mono-unsaturated fatty acids.
[c] n-6 PUFA: n-6 poly-unsaturated fatty acids.
[d] n-3 PUFA: n-3 poly-unsaturated fatty acids.
[e] LC-PUFA: long chain-polyunsaturated fatty acids.
[f] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C20:0, C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C18:3n-6, C20:3n-6, C18:4n-3, C22:5n-3, are not listed in the table. Data are presented as means ± S.E.M. Means in each row sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means.

Table 7
Plasma biochemical indexes and enzyme activities of large yellow croaker (*Larmichthys crocea*) fed diets with graded levels of fish oil replaced by palm oil (Means ± S.E.M.)[g].

Plasma biochemical indexes	0% (the control)	33.3%	66.7%	100%
TC[a] (mmol/L)	2.69 ± 0.19[b]	2.76 ± 0.22[b]	2.85 ± 0.10[ab]	3.61 ± 0.30[a]
TG[b] (mmol/L)	3.28 ± 0.58	4.78 ± 0.89	4.20 ± 0.41	4.93 ± 0.31
HDL-C[c] (mmol/L)	0.77 ± 0.06[a]	0.86 ± 0.05[a]	0.70 ± 0.02[a]	0.50 ± 0.05[b]
LDL-C[d] (mmol/L)	0.71 ± 0.08[c]	0.76 ± 0.05[bc]	1.06 ± 0.07[ab]	1.17 ± 0.11[a]
ALT[e] (U/L)	56.11 ± 2.70[b]	57.93 ± 2.07[b]	69.78 ± 2.60[a]	75.59 ± 4.02[a]
AST[f] (U/L)	5.33 ± 0.55[b]	5.42 ± 0.53[b]	6.11 ± 0.36[ab]	7.44 ± 0.33[a]

[a] TC: Total cholesterol.
[b] TG: Triglyceride.
[c] HDL-C: High density lipoprotein cholesterol.
[d] LDL-C: Low density lipoprotein cholesterol.
[e] ALT: Alanine transaminase.
[f] AST: Aspartate aminotransferase.
[g] Data are presented as means ± S.E.M. Means in each row sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means.

group ($P > 0.05$) (Fig. 4). Moreover, dietary PO induced a significantly increasing ratio of n-p65 to t-p65 protein, which demonstrated the activation of NF-κB signaling ($P < 0.05$).

4. Discussion

In the present study, high levels of PO in diets impaired growth performance and promoted lipid deposition in the liver of large yellow croaker. Specific growth rate and feed efficiency ratio were significantly decreased in fish fed the diet with 100% PO compared to the control group. This was probably due to the imbalance of fatty acid in diets since dietary SFA increased with increasing dietary PO levels. Therefore, the excessive SFA could decrease FA absorption [29,30], which could be responsible for the decreased growth rate and feed efficiency of fish fed the diet with 100% PO. Furthermore, SFA has been reported to promote lipid deposition [31], which resulted in the increase of lipid content in the liver of large yellow croaker. This result was also in agreement with findings in African catfish [32], gilthead sea bream [33] and rainbow trout (*Oncorhynchus mykiss*) [34].

In order to evaluate the physiological state of large yellow croaker, plasma biochemical indexes were analyzed. Concerning plasma lipid, the increase of dietary PO levels was accompanied with increased contents of serum LDL-C and TC and the decreased content of HDL-C in fish (Table 7). Similarly, the increase of dietary PO levels significantly increased the contents of serum LDL-C in Nile tilapia (*Oreochromis niloticus*) [35], which indicated that fish were in hyperlipidemic status when fish were fed the diet with high dietary PO levels. However, further investigations are required to understand the exact mechanisms on how the increase of dietary PO levels cause the increased concentration of serum TC and LDL-C. The liver is a pivotal organ of the nutrient metabolism, and the health status of the liver is one of important evaluation factors of physiological status. In order to evaluate the healthy status of the liver, activities of the serum AST and ALT were determined as biomarkers. Commonly, the higher activities of serum

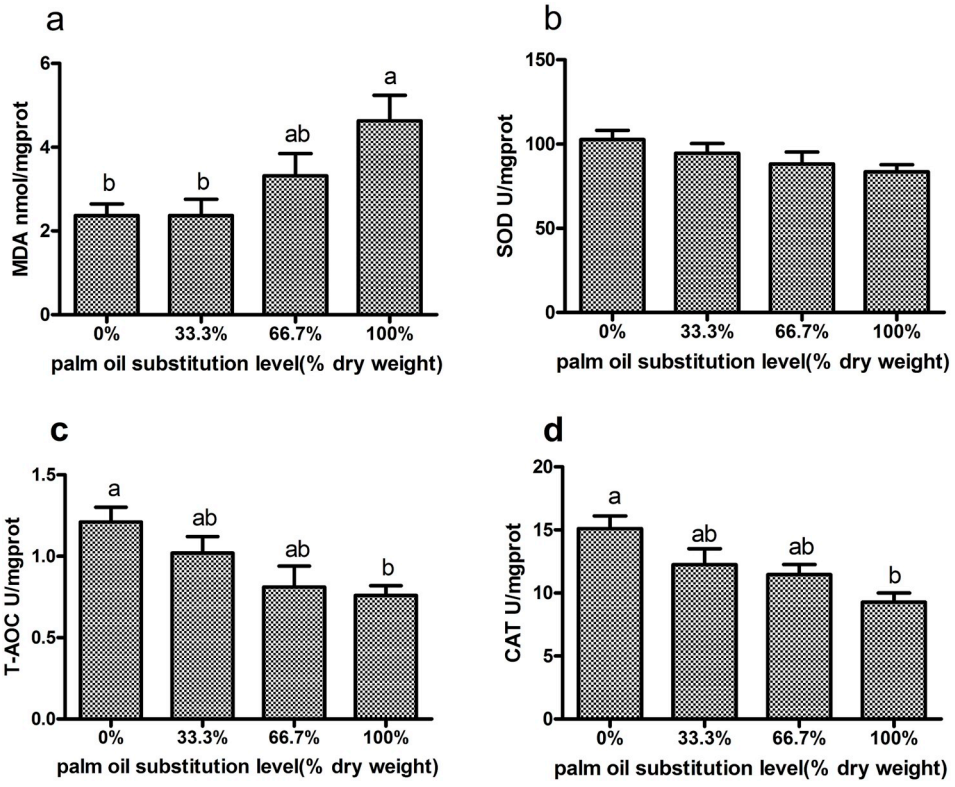

Fig. 1. Enzyme indexes (a)MDA (b)SOD (c)T-AOC (d)CAT in the liver of large yellow croaker (*Larmichthys crocea*) fed diets with graded levels of fish oil replaced by palm oil. Enzyme indexes are expressed as nmol or U per milligram protein. MDA: malondialdehyde, SOD: superoxide dismutase, T-AOC: total antioxidant capacity, CAT: catalase. 0%: control diet with 0% fish oil replaced by palm oil; 33.3%: diet with 33.3% fish oil replaced by palm oil; 66.7%: diet with 66.7% fish oil replaced by palm oil; 100%: diet with fish oil totally replaced by palm oil; Data are presented as means ± S.E.M. Means in each bar sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means.

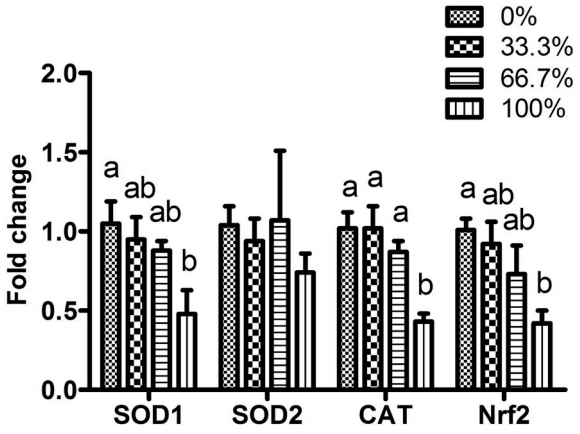

Fig. 2. Antioxidant related gene expression in the liver of large yellow croaker (*Larmichthys crocea*) fed diets with graded levels of fish oil replaced by palm oil. 0%: control diet with 0% fish oil replaced by palm oil; 33.3%: diet with 33.3% fish oil replaced by palm oil; 66.7%: diet with 66.7% fish oil replaced by palm oil; 100%: diet with fish oil totally replaced by palm oil; Data are presented as means ± S.E.M. Means in each bar sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means.

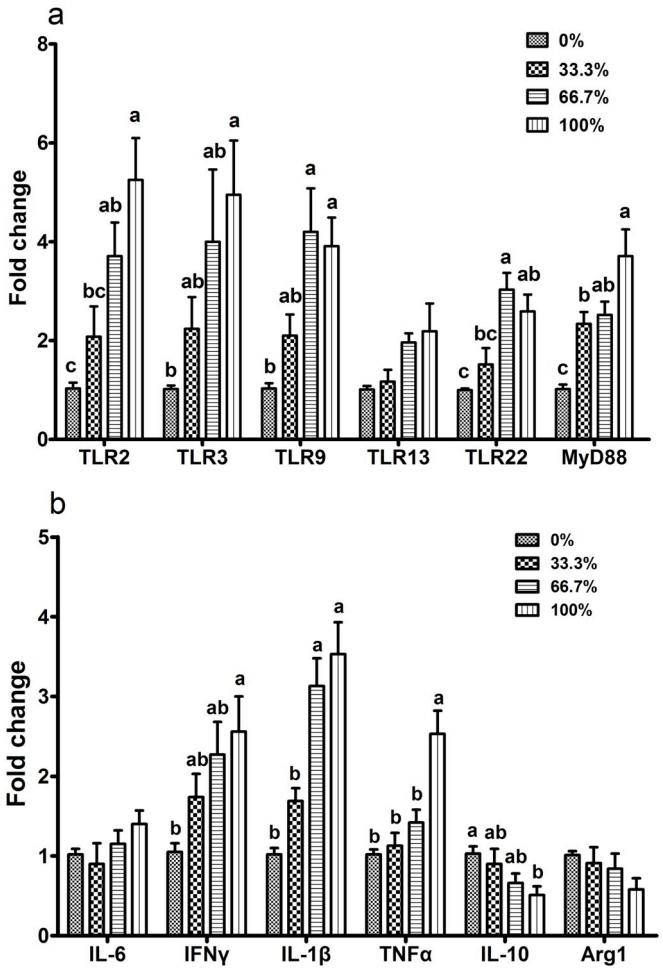

Fig. 3. Expression of genes related to (a) the TLR signaling pathway and (b) the inflammation in large yellow croaker (*Larmichthys crocea*) fed diets with graded levels of fish oil replaced by palm oil. 0%: control diet with 0% fish oil replaced by palm oil; 33.3%: diet with 33.3% fish oil replaced by palm oil; 66.7%: diet with 66.7% fish oil replaced by palm oil; 100%: diet with fish oil totally replaced by palm oil; Data are presented as means ± S.E.M. Means in each bar sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means.

AST and ALT reflect the more severity of liver damage [36]. In this study, activities of serum AST and ALT were increased with increasing dietary PO levels, which was similar to the results of tilapia (*Oreochromis niloticus*) [35] and zebrafish (*Danio rerio*) [37]. Results of plasma biochemical indexes suggested that fish fed the diet with 100% PO were not in relatively healthy status.

Previous investigations have indicated that high inclusion of dietary PO decreased the antioxidant capacity of obese individuals [10]. In this study, high PO inclusion in diets reduced the SOD1 and CAT mRNA expression as well as T-AOC, activities of CAT, which were essential to scavenge the toxic intermediate of incomplete oxidation. Furthermore, the content of MDA in fish fed the diet with 100% PO was significantly increased compared to the control group. Nrf2 is a redox-sensitive transcription factor, which plays a central role in cellular defense against oxidative and electrophilic insults to maintain antioxidant capacity [38]. In this study, the Nrf2 mRNA expression was decreased with the increase of dietary PO levels. Similar result have been found in Japanese sea bass that high percentage of dietary vegetable oil decreases the antioxidant capacity through the Nrf2 pathway [39]. Thus, 100% PO in the diet decreased the antioxidant capacity of fish probably through the Nrf2 pathway.

The decreased antioxidant capacity had been proven to be related with the increased inflammation [40–42]. Further, both the decreased antioxidant capacity and the increased inflammation cause injury to organism [43], which could be responsible for the rough physiological state of fish fed the diet with 100% PO in this study. Previous studies have demonstrated that SFA could stimulate the TLR4/NF-κB pathway to trigger the inflammation [44,45]. In the present study, the TLR2, TLR3, TLR9, TLR22, MyD88 mRNA expression and pro-inflammatory gene expression were increased with increasing dietary PO levels. The ratio of n-p65 to t-p65 protein was significantly up-regulated in the liver of juvenile fish fed the diet with 100% PO compared to the control group, which suggested the activation of TLR-NF-κB signaling pathway. Evidence in literature have reported that the activation of TLR-NF-κB signaling pathway lead to the increased IL-1β and TNFα mRNA expression [14]. Those results were similar to one of our previous findings that high lipid diet activated the TLR-NF-κB signaling pathways and induced cytokine expression in turbot [46]. The mRNA expression of IL-10 was significantly decreased when fish were fed the diet with 100% PO compared to the control group. IL-10 was generally taken as an anti-inflammatory cytokine, and its production could be regulated by fatty acid to exert protection against over-activation of immunological pathways [47,48]. Compared to the untreated cells, murine 3T3-L1 adipocytes exhibited a 75% decrease of IL-10 production with palmitic acid incubation for 24 h [49]. Furthermore, studies have reported that activated NF-κB could suppress the expression or activity of anti-inflammatory cytokines like IL-10 and TGFβ [50]. Therefore, compared to the control group, the induced inflammatory response in large yellow croaker fed the diet with 100% PO might be due to the activation of the TLR-NF-κB signaling.

In conclusion, high percentage of dietary PO suppressed the growth and antioxidant capacity and induced the inflammation by the activation of TLR-NF-κB signaling pathway in large yellow croaker.

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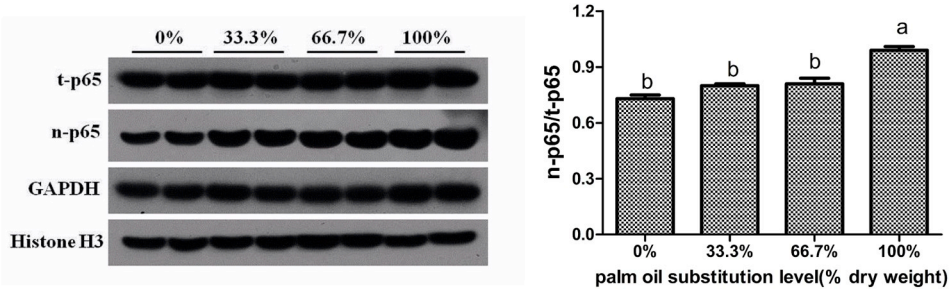

Fig. 4. Western blot analyses for NF-κB signaling activation in the liver of large yellow croaker. The right panel features the ratio of n-p65 to t-p65. GAPDH and Histone H3 are selected as total and nucleus reference proteins, respectively. Data are expressed as the A.U. of the Western blot. 0%: control diet with 0% fish oil replaced by palm oil; 33.3%: diet with 33.3% fish oil replaced by palm oil; 66.7%: diet with 66.7% fish oil replaced by palm oil; 100%: diet with fish oil totally replaced by palm oil; Data are presented as means ± S.E.M. Means in each bar sharing the same superscript letter or absence of superscripts are not significantly different determined by Tukey's test ($P > 0.05$). S.E.M.: standard error of means. NF-kB: nuclear factor kappa beta; t-p65: total p65; n-p65: nucleus p65; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

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