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# Dietary vegetable oil suppressed non-specific immunity and liver antioxidant capacity but induced inflammatory response in Japanese sea bass (*Lateolabrax japonicus*)

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ABSTRACT

High percentage of dietary vegetable oil (VO) induced negative effects on immunity in numerous fish species. The present study was conducted to investigate whether VO could exert anti-immunological effects by regulating non-specific immunity, liver antioxidant capacity and nuclear factor kappa beta (NF-κB) signaling in Japanese sea bass (*Lateolabrax japonicus*). Three iso-nitrogenous and iso-lipid diets were formulated by replacing 0% (FO, the control), 50% (FV) and 100% (VO) of fish oil with vegetable oil. Each diet was randomly fed to triplicate groups of fish for 10 weeks. Results showed that the alternative complement pathway (ACP) activity and the disease resistance were significantly lower in fish fed VO diets compared with the control group ($P < 0.05$). Liver superoxide dismutase (SOD), catalase (CAT) and glutathion peroxidase (GPx) enzyme activities, as well as total antioxidant capacity (T-AOC) significantly decreased in fish fed VO diets ($P < 0.05$). Meanwhile, significantly low level of liver SOD1 and CAT mRNA, nuclear factor erythroid 2-related factor 2 (Nrf2) of both mRNA and protein were observed in fish fed VO diets when compared with fish fed FO diets ($P < 0.05$). However, the transcription level of TNFα and IL1β was significantly higher in the liver of fish fed VO diets, which might be attributed to the activation of NF-κB signaling pathway since the protein expression of p65, one of the key members of NF-κB family, was significantly increased ($P < 0.05$). These results suggested that dietary VO could lower the ACP activity, disease resistance and liver antioxidant capacity, but it could also exacerbate inflammatory response by activating NF-κB signaling pathway in Japanese sea bass.



## 1. Introduction

Aquaculture provides a major source of long chain-polyunsaturated fatty acid (LC-PUFA) for human consumption and trends indicate that it will play a much more important role since the natural fishery resources have been exploited at their maximum sustainable limit [1]. Fish oil (FO) is usually the major lipid component in fish diets. However, limited FO output cannot meet the increasing demand of aqua-feed industry [2]. In consequence, investigation of FO alternatives has become a hot research topic. The possibility of partial or total replacement of dietary FO by vegetable oil (VO) without affecting fish growth was reported in European sea bass (*Dicentrarchus labrax*) [3,4], gilthead sea bream (*Sparus aurata*) [5] and Atlantic Salmon (*Salmo salar*) [6,7]. However, excessive dietary VO inclusion has negative effects on non-specific immunity in Rainbow trout (*Oncorhynchus mykiss*) [8], gilthead sea bream [9] and Atlantic salmon [10].

Proper nutrition is essential not only to achieve optimal growth rate but also to maintain fish health [11]. Therefore, numerous investigations have been conducted from the perspective of membrane fluidity, eicosanoid pathways [12], pattern recognition receptor pathways [13], interferon system [14] and antioxidant system [15] to elucidate the mechanism of VO in inducing negative effects in fish during the past years.

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Alvarez et al. [16] reported that the antioxidant defense system includes enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and other low molecular weight scavengers in fish. The antioxidant system is crucial to fish immunity by eliminating the reactive oxygen species (ROS) and reactive nitrogen species (RNS). Previous studies in large yellow croaker (*Larmichthys crocea*) [13], Atlantic salmon [17], Rainbow trout [15,18] and Japanese sea bass (*Lateolabrax japonicus*) [19], have demonstrated that appropriate dietary n-3 LC-PUFA content is beneficial to improve fish antioxidant capacity or relieve oxidative stress. Moreover, administration of FO enhanced antioxidant system performance yet decreased the content of hydroperoxides in a rabbit model [20]. Similar results were reported by Kirimlioglu [21] and Kasdallah-Grissa [22] in rats. Mechanism of dietary FO involved in improving antioxidant performance was suggested to be related to Nrf2 signaling pathway [23], which modulates the transcription of type II detoxifying enzyme genes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) in fish [24]. Previous study in grass carp (*Ctenopharyngodon idella*) indicated dietary alpha-linolenic acid/linoleic acid ratios modulate SOD, GPx, glutathione *S*-transferase (GSH) and glutathione reductase (GR) activities and mRNA levels, as well as signaling molecule nuclear factor Nrf2 gene transcriptional abundance in the intestine [25]. However, the mechanism of lipid sources or fatty acids involved in modulating antioxidant system is still unclear in fish species.

Different lipid sources exerted different effects on inflammatory response. VO was found to induce the expression of pro-inflammatory cytokine genes in Senegalese sole (*Solea senegalensis*) [26], Japanese Flounder (*Paralichthys olivaceus*) [27] and gilthead sea bream [28]. In contrast, FO inhibited the production of pro-inflammatory cytokines by suppressing the NF-κB signaling pathway in mammals [29]. However, the role of NF-κB signaling pathway in regulating inflammatory response which was induced by dietary FO replaced by VO has not been extensively studied in Japanese sea bass and other fish species.

The object of this study was to investigate the effects of dietary VO on non-specific immunity, liver antioxidant capacity and NF-κB signaling in Japanese sea bass, which will be beneficial to the health of reared fish based on the better understanding of its immune-modulatory mechanism.

## 2. Materials and methods

### 2.1. Animals, diets formulation and animal husbandry

Animals, diets formulation and animal husbandry were described as previous study [30]. Briefly, disease-free and equal sizes Japanese sea bass were obtained from a commercial farm. Three iso-nitrogenous (41% crude protein) and iso-lipid (12% crude lipid) diets with 0% (FO), 50% (FV, fish oil: linseed oil: soybean oil = 2:1:1) and 100% (VO, linseed oil: soybean oil = 1:1) vegetable oil were formulated and the proximate composition was analyzed (Table 1). The content of different fatty acids in the experimental diets (Table 2) was measured as previous study [30]. Animal experiment was carried out in a net cage system at Xihu harbor (Ningbo, China). Fasted for 24 h, Japanese sea bass (18.60 ± 0.36 g) were randomly divided into to nine floating cages (30 fish/cage). Each kind of diet was randomly divided into three parts and each was randomly assigned to a net cage. Fish were fed twice per day to apparent satiation for 10 weeks. Husbandry of Japanese sea bass were under appropriate conditions. The protocols for animal husbandry and handling employed in this study were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

### 2.2. Sample collections

At the end of the feeding trial, nine fish per cage was randomly collected and anaesthetized with MS222 (Sigma, USA). Blood samples were collected from the caudal vasculature and put still at

**Table 1**
Formulation of the experimental diets (% dry matter) [30].

| Ingredients | FO[a] | FV[b] | VO[c] |
|---|---|---|---|
| Defatted white fish meal[d] | 15 | 15 | 15 |
| Soybean meal | 32 | 32 | 32 |
| Casein[e] | 11 | 11 | 11 |
| Wheat meal | 26 | 26 | 26 |
| Mineral premix[f] | 2 | 2 | 2 |
| Vitamin premix[g] | 2 | 2 | 2 |
| Attractantd[h] | 0.3 | 0.3 | 0.3 |
| Mold inhibitor[i] | 0.1 | 0.1 | 0.1 |
| Lecithin | 2.6 | 2.6 | 2.6 |
| Fish oil | 9 | 4.5 | 0 |
| Soybean oil | 0 | 2.5 | 4.5 |
| Linseed oil | 0 | 2.5 | 4.5 |
| Total | 100 | 100 | 100 |
| Dry % | | | |
| Crude protein | 41.67 | 41.74 | 41.71 |
| Crude lipid | 12.85 | 12.70 | 12.76 |

[a] FO: Fish oil group.
[b] FV: blend vegetable oil (linseed oil/soybean oil = 1:1) replacement fish oil with 50%.
[c] VO: blend vegetable oil replacement fish oil with 100%.
[d] Defatted fish meal: 72.1% crude protein and 1.4% crude lipid; white fish meal were defatted with ethanol (fish meal: ethanol = 1:2 (w:v)) at 37°C for three times.
[e] Casein: 88% crude protein and 1.3% crude lipid, Alfa Aesar, Avocado Research Chemicals Ltd, UK.
[f] Mineral premix (mg or g $kg^{-1}$ diet): $CuSO_4 \cdot 5H_2O$ 10 mg; $Na_2SeO_3$ (1%) 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1%) 50 mg; $MnSO_4 \cdot H_2O$ 60 mg; $FeSO_4 \cdot H_2O$ 80 mg $Ca(IO_3)_2$ 180 mg; $MgSO_4 \cdot 7H_2O$ 1200 mg; zeolite 18.35 g.
[g] Vitamin premix (mg or g $kg^{-1}$diet): vitamin D 5 mg; vitamin K 10 mg; vitamin B12 10 mg; vitamin B6 20 mg; folic acid 20 mg; vitamin B1 25 mg; vitamin A 32 mg; vitamin B2 45 mg; pantothenic acid 60 mg; biotin 60 mg; niacin acid 200 mg; α-tocopherol 240 mg; inositol 800 mg; ascorbic acid 2000 mg; microcrystalline cellμ lose 16.47 g.
[h] Phagostimulant: Glycine/Betaine = 1:3.
[i] Preservative: Fumarate/Calcium propionate = 1:1.

**Table 2**
The content of different fatty acids in the experimental diets (mg/g)[a] [30].

| Fatty acid | FO | FV | VO |
|---|---|---|---|
| C 14: 0 | 0.76 | 0.42 | 0.10 |
| C 16: 0 | 4.51 | 3.96 | 3.13 |
| C 18: 0 | 1.63 | 1.72 | 1.71 |
| ∑SFA[b] | 6.90 | 6.10 | 4.94 |
| C 16: 1 | 1.08 | 0.53 | 0.06 |
| C 18: 1 | 3.59 | 4.58 | 5.47 |
| ∑MUFA[c] | 4.67 | 5.11 | 5.53 |
| C 18: 2n-6 | 4.35 | 8.85 | 12.66 |
| C 20: 4n-6 | 0.12 | 0.07 | 0.04 |
| ∑n-6 PUFA[d] | 4.47 | 8.93 | 12.70 |
| C 18: 3n-3 | 0.43 | 3.32 | 6.98 |
| C 20: 5n-3 | 1.25 | 0.62 | 0.06 |
| C 22: 6n-3 | 1.85 | 0.88 | 0.08 |
| ∑n-3 PUFA[e] | 3.53 | 4.82 | 7.12 |
| ∑n-3/∑n-6 PUFA | 0.79 | 0.54 | 0.56 |
| ∑n-3 LC-PUFA | 3.10 | 1.49 | 0.14 |
| Total fatty acids | 21.18 | 26.82 | 31.09 |

[a] The fatty acid composition was determined by Gas Chromatograph Mass Spectrometer (GC–MS). A Thermo TRACE 1310 GC-ISQ QD MS mass spectrometer equipped with an Agilent 7890AGC-5975CMS gas chromatograph was employed. Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22: 0, C24: 0, C14: 1, C20: 2n-6, C20:3n-6, were not listed in the table.
[b] SFA: saturated fatty acid.
[c] MUFA: monounsaturated fatty acid.
[d] n-6 PUFA: n-6 poly-unsaturated fatty acid.
[e] n-3 PUFA: n-3 poly-unsaturated fatty acid.

**Table 3**
Liver fatty acids content of Japanese sea bass fed the experimental diets with vegetable oil instead of fish oil (mg/g, Means ± SE)[a] [30].

| Fatty acid | FO[b] | FV[c] | VO[d] |
|---|---|---|---|
| C 14: 0 | 1.42 ± 0.17 | 1.56 ± 0.03 | 1.70 ± 0.06 |
| C 16: 0 | 8.57 ± 0.04b | 8.42 ± 0.33b | 10.36 ± 0.37a |
| C 18: 0 | 4.25 ± 0.11b | 4.65 ± 0.23b | 6.81 ± 0.17a |
| ∑SFA[e] | 14.25 ± 0.32b | 14.64 ± 0.56b | 18.87 ± 0.48a |
| C 16: 1 | 2.33 ± 0.18 | 2.35 ± 0.07 | 2.30 ± 0.13 |
| C 18: 1 | 10.41 ± 0.31c | 23.23 ± 0.15b | 32.08 ± 0.61a |
| ∑MUFA[f] | 12.74 ± 0.49c | 25.58 ± 0.22b | 34.27 ± 0.68a |
| C 18: 2n-6 | 3.33 ± 0.29b | 2.66 ± 0.35b | 5.40 ± 0.39a |
| C 20: 4n-6 | 0.10 ± 0.00a | 0.06 ± 0.01b | 0.03 ± 0.00c |
| ∑n-6 PUFA[g] | 3.43 ± 0.29b | 2.71 ± 0.36b | 5.45 ± 0.38a |
| C 18: 3n-3 | 0.48 ± 0.08b | 0.70 ± 0.08 ab | 0.84 ± 0.06a |
| C 20: 5n-3 | 0.20 ± 0.01a | 0.06 ± 0.01b | 0.03 ± 0.00c |
| C 22: 6n-3 | 0.65 ± 0.03a | 0.16 ± 0.03b | 0.07 ± 0.01b |
| ∑n-3 PUFA[h] | 1.33 ± 0.12a | 1.34 ± 0.22a | 0.94 ± 0.05b |
| ∑n-3/∑n-6 PUFA | 0.39 ± 0.00a | 0.34 ± 0.03a | 0.17 ± 0.02b |
| ∑n-3 LC-PUFA | 0.85 ± 0.03a | 0.22 ± 0.04b | 0.10 ± 0.01b |
| Total fatty acids | 34.05 ± 1.37c | 47.37 ± 1.30b | 62.20 ± 0.91a |

Values in the same row with no common superscript letters are significantly different ($P < 0.05$). Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22: 0, C24: 0, C14: 1, C20: 2n-6, C20:3n-6, were not listed in the table.

[a] The fatty acid composition was determined by Gas Chromatograph Mass Spectrometer (GC–MS). A Thermo TRACE 1310 GC-ISQ QD MS mass spectrometer equipped with an Agilent 7890AGC-5975CMS gas chromatograph was employed.

[b] FO: 100% Fish oil as lipid source (control) in Japanese sea bass.

[c] FV: Vegetable oil blend (linseed oil:/soya bean oil = 1:1) replacing 50% of fish oil in Japanese sea bass.

[d] VO: 100% Vegetable oil blend as lipid source in Japanese sea bass.

[e] SFA: saturated fatty acid.

[f] MUFA: monounsaturated fatty acid.

[g] n-6 PUFA: n-6 poly-unsaturated fatty acid.

[h] n-3 PUFA: n-3 poly-unsaturated fatty acid.

4 °C for 6 h. After centrifugation (3000×g, 10 min, 4 °C), the clot was discarded and blood cells were separated from the residual straw-colored serum. Liver tissues were then collected from the nine fish of each cage on ice pack. All samples were immediately frozen in liquid nitrogen and stored at −80 °C prior to analysis. Determination of liver fatty acids content of Japanese sea bass (Table 3) fed the experimental diets with vegetable oil instead of fish oil was described as previous study [30].

### 2.3. Head kidney macrophages separation and respiratory burst activity assay

Head kidney macrophages were separated as described by previous study with some modifications [31]. Six to nine fish were randomly collected and anaesthetized with MS222 (Sigma, USA). Fish were dissected to obtain the head kidneys. Head kidneys were washed with L-15 medium (Sigma, USA), and forced to pass through 100 μm cell strainer (Falcon, USA) into 50 mL centrifuge tube (Corning, USA). The L-15 medium was formulated with 100 U penicillin and streptomycin, 2 mM L-alanyl-L-glutamine (Thermo Fisher Scientific, USA) and 2% fetal bovine serum (Gibco, USA). Head kidney leucocyte suspensions were enriched by centrifugation (836 ×*g* for 25 min at 4 °C) for macrophages using a 34–51% Percoll density gradient. Separated macrophages were countered in a haemocytometer and modulated to $1 \times 10^7 mL^{-1}$. Cell viability was guaranteed to be more than 95% for further experiment.

Head kidney macrophages respiratory burst activity was measured by the nitro-blue-tetrazolium (NBT) (Sigma, USA) assay, described as previous study with some modifications [31]. Head kidney macrophages suspension (100 μL, $1 \times 10^7$/mL) was added into a 96 well cell culture plate to centrifuge for 10 min (1500×*g*). Supernatant was then replaced by 200 μL fresh L-15 medium (NBT, Sigma, USA, 1 mg/mL; Phorbol 12-myristate 13-acetate, PMA, Sigma, USA, 1 μg/mL). Cells were fixed after incubation (40 min, 18 °C, in dark) by 200 μL methanol per well. Subsequently, each well was washed with 70% methanol aqueous solution and incubated for 10 min. Each well was washed twice and then air dried. Blue precipitation formed in the well was dissolved with 120 μL potassium hydroxide aqueous solution (2 M) and DMSO (Sinopharm Chemical Reagent, China). The respiratory burst activity was expressed as absorbance value detected under 630 nm wave length.

### 2.4. Serum lysozyme activity

The lysozyme activity in serum was measured according to previous study [32]. Briefly, 0.05 ml serum was added into 1.4 ml *Micrococcus lysodeikticus* (Sigma, USA) PBS suspension (0.2 mg $ml^{-1}$). The reaction was carried out at 25 °C and absorbance was measured after 0.5 and 4.5 min by UV–vis recording spectrophotometer (Shimadzu, Japan) at 530 nm wavelength. One unit was defined as the amount of serum causing a decrease of 0.001/min in absorbance at 530 nm.

### 2.5. Alternative complement pathway (ACP) activity

Serum ACP activity for nine fish in each cage was assayed according to the method previously described [33]. A lysis curve was created to determine the volume of serum that yielded 50% hemolysis and the number of ACH50 units $mL^{-1}$ was obtained for each group.

### 2.6. Immune challenge with Vibrio anguillarum

After the sample collection, Japanese sea bass in the original net cage (10 fish per net cage) were anaesthetized and transferred to new net cages (1 m*1 m*1 m) in a cement pool (6 m*5 m*2 m) for immune challenged with *Vibrio anguillarum*. Bacteria was prepared referring to the procedures described in a previous study [34]. Right before the immune challenge, the *V. anguillarum* culture was suspended in phosphate-buffer saline and kept on ice. Half lethal concentration ($1.0 \times 10^9$ CFU) of *V. anguillarum* was injected into the enterocoelia of each fish. After injection, the fish were returned to their original cages. Mortality was monitored in the next

**Table 4**
Primers usedfor q-PCR in this study.

| Primer names | Forward and reverse primer sequences (5′ to 3′) | Tm (°C) | PCR efficiency (%) |
|---|---|---|---|
| Nrf2-F/R | AGAAGGAGCGTCTGTTGAGTGA/GGAAGATGCTGCCGTTAGTTGA | 60 | 97 |
| SOD1-F/R | AGAATCATGCCGGTCCTAATG/CGGTGATGTCTATCTTGGCTAC | 59 | 96 |
| CAT-F/R | TGTGGGACTTCTGGAGCCTGAG/TGTGAGAGCCGTAGCCGTTCAT | 59 | 99 |
| IL1β-F/R | TGGAATCCCAGATGAAATGC/GTTGAACTGCGCTCAAACAC | 60 | 102 |
| TNFα-F/R | CGCTACTCGGACTCCATAGGC/CTGAAACACCGCACCCAGATA | 62 | 99 |
| βactin-F/R | CAACTGGGATGACATGGAGAAG/TTGGCTTTGGGGTTCAGG | 58 | 101 |

Nrf2: nuclear factor erythroid 2-related factor 2, SOD: superoxide dismutase, CAT: catalase, IL1β: interleukin 1β, TNFα: tumor necrosis factor α.

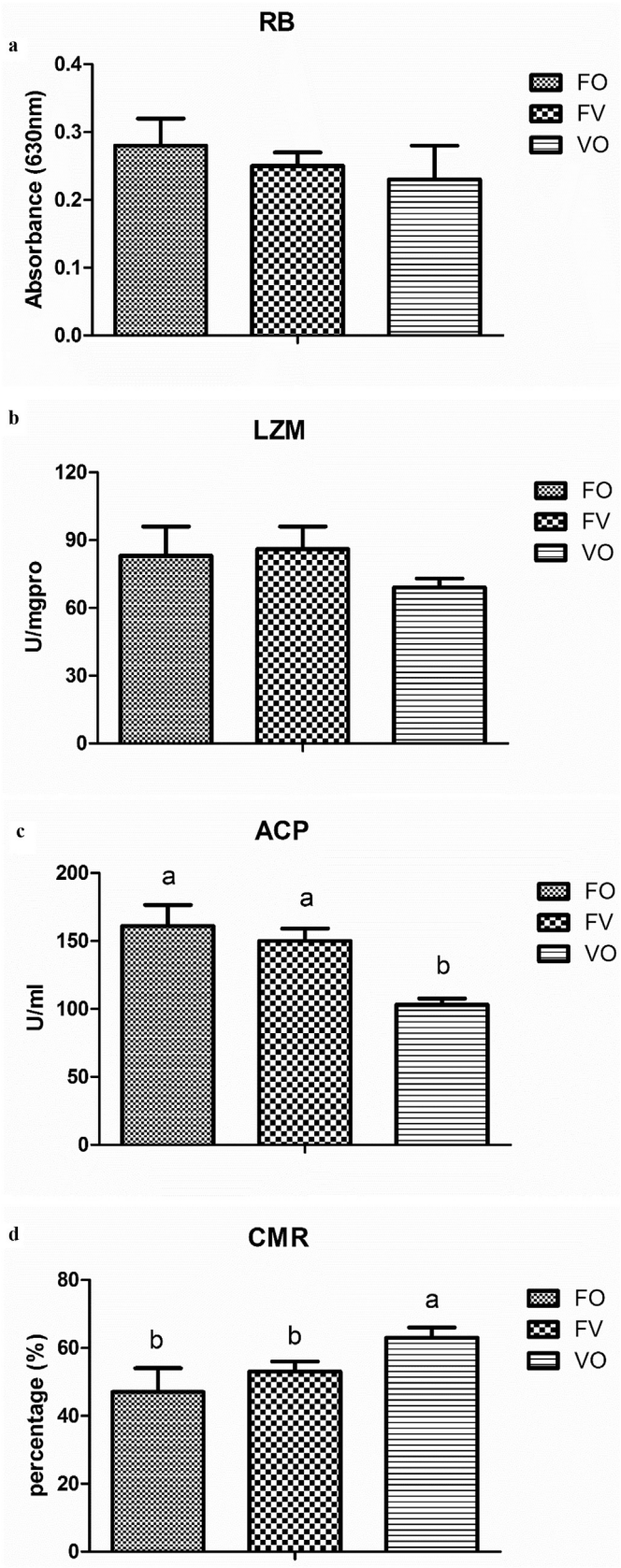


**Fig. 1. VO decreased non-specific immunity parameters and disease resistance in Japanese sea bass.** Head kidney macrophages respiratory burst (RB) activity (Fig. 1a), serum lysozyme (LZM) activity (Fig. 1b), alternative complement pathway (ACP) activity (Fig. 1c) and 7-day cumulative mortality rate (CMR) are presented when fish was fed different diets. FO: control diet, with 0% vegetable oil inclusion; FV: diet with 50% vegetable oil and 50% fish oil; VO: dietary fish oil totally replaced by vegetable oil. Values are means ± S.E.M of three replicates. Different letters in each figure presents significant difference among dietary treatments by Tukey's test ($P < 0.05$). S.E.M.: stand error of means.

following 7 days. After the immune challenge, dead fish and sea water in the pool were both collected and strict processed, to avoid the release of the pathogenic bacteria to the seawater.

### 2.7. Antioxidant enzyme activities in liver tissue

Before analysis, tissues were ground into powder in liquid nitrogen, homogenized in PBS and centrifuged at 4000×*g* for 10 min. Protein concentration in supernatant was determined using a Bradford Protein Assay Kit (Beyotime Institute of Technology, China). Activities of liver superoxide dismutase (SOD), catalase (CAT), glutathion peroxidase (GPx) and total antioxidant capacity (T-AOC) were determined using commercial kits (Nanjing Bioengineering Institute, Nanjing, China).

### 2.8. RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction (q-PCR)

Liver tissues were ground in liquid nitrogen environment and total RNA was extracted using Trizol reagent (Takara, China) following the manufacturer's protocol. To remove the genome DNA, roughly extracted RNA was incubated with DNase (Takara, China) at 42 °C. The integrity of RNA was measured by electrophoresis on 1.2% denatured agarose gel and the quantity on Nano Drop® 2000 spectrophotometer (Thermo fisher scientific, USA). Total RNA of 260/280 nm absorbance ratio of 1.8–2.0 was used for further experiments. The extracted RNA was reversely transcribed to first-strand cDNA using Primer Script ™ RT reagent Kit (Takara, China) under the manufacturer's instructions.

Real-time polymerase chain reaction was performed as previously described [35]. Each sample in the reaction was performed with three duplicates. Primers were designed following the published sequences (Table 4). To calculate the relative expression of genes, the comparative CT method ($2^{-\triangle\triangle CT}$ method) was adopted, and the value stood for *n*-fold difference relative to the calibrate [36].

### 2.9. Western blot

Nuclear protein of liver tissue was extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Total liver tissue proteins were extracted according to the previous description [37]. Protein concentration was quantified using a Bradford Protein Assay Kit (Beyotime Institute of Technology, China). Equal amounts of protein were loaded into the wells and then separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis. Proteins in the gel were transferred to activated polyvinylidene fluoride (PVDF) membrane (Millipore, USA). PVDF membrane was incubated overnight with primary antibody and then washed with tris buffered saline with tween (TBST) three times, 5 min each time. Membrane was then incubated for 1 h with horseradish peroxide (HRP)-conjugated secondary antibody. Immune complexes were visualized using an Electrochemiluminescence (ECL) Kit (Beyotime Institute of Technology, China).

Polyclonal Lamin B was obtained from Santa Cruz Biotechnology (USA). Nrf2 was obtained from Abcam (UK), whereas *anti*-p65

antibody was purchased from Cell signaling Technology (USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and HRP-conjugated secondary antibodies were from Golden Bridge Biotechnology (China).

### 2.10. Calculations and statistical analysis

Cumulative mortality rate $= N_f \times 100/N_i$ $N_f$ is the final number of fish left in each cage after immune challenge and $N_i$ is the initial number of fish in each cage before the immune challenge ($N_i = 10$).

Statistical analysis was performed in SPASS 20.0 (SPASS Inc., USA). Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's test. For statistically significant differences, $P < 0.05$ was required. Results were presented as mean ± S.E.M (standard error of means).

## 3. Results

### 3.1. Non-specific immunity parameters and disease resistance

No significant difference was observed in head kidney macrophages respiratory burst (RB) activity and serum lysozyme (LZM) activity among fish fed different diets ($P > 0.05$) (Fig. 1a and Fig. 1b). However, alternative complement pathway (ACP) activity significantly decreased in fish fed VO diets ($P < 0.05$) (Fig. 1c). Seven-day immune challenge cumulative mortality rate (CMR) increased significantly ($P < 0.05$) in fish fed VO diets though no statistically significant difference was observed between fish fed FO and FV diets ($P > 0.05$) (Fig. 1d).

### 3.2. Antioxidant enzyme activities

Total SOD, CAT, GPx and T-AOC activities in the liver of fish fed VO diets significantly decreased compared with FO group ($P < 0.05$). No significant difference of total SOD activity was found between the FO and FV groups ($P > 0.05$) (Fig. 2a). Liver CAT, GPx and T-AOC activity showed no significant difference either between the FO and FV groups or between the FV and VO groups ($P > 0.05$) (see Fig. 3).

### 3.3. Antioxidant (SOD1, CAT and Nrf2) and cytokine (IL1β and TNFα) related genes expression

The lowest expression levels of SOD1, CAT and Nrf2 mRNA were observed in the liver of fish fed VO diets, which were significantly lower than the control group ($P < 0.05$). Relative expression of IL1β and TNFα both increased significantly in the liver of fish fed VO diets when compared with fish fed FO diets ($P < 0.05$) (see Fig. 4).

### 3.4. Western blots for Nfr2 and NF-κB

The ratio of total Nrf2 to GAPDH decreased with the replacement of dietary FO by VO though there was no statistically significant difference among the treatments ($P > 0.05$). Nucleus Nrf2 amount in liver of fish fed VO diets was significantly lower than that of fish fed FO and FV diets ($P < 0.05$). Meanwhile, the amount of nucleus Nrf2 was significantly higher in liver of fish fed FO diets than that of fish fed FV diets ($P < 0.05$). No significant difference of total p65 protein amount was found among all treatments. However, dietary VO induced a significant over-expression of nucleus p65 protein ($P < 0.05$), which indicated the activation of NF-κB signaling.

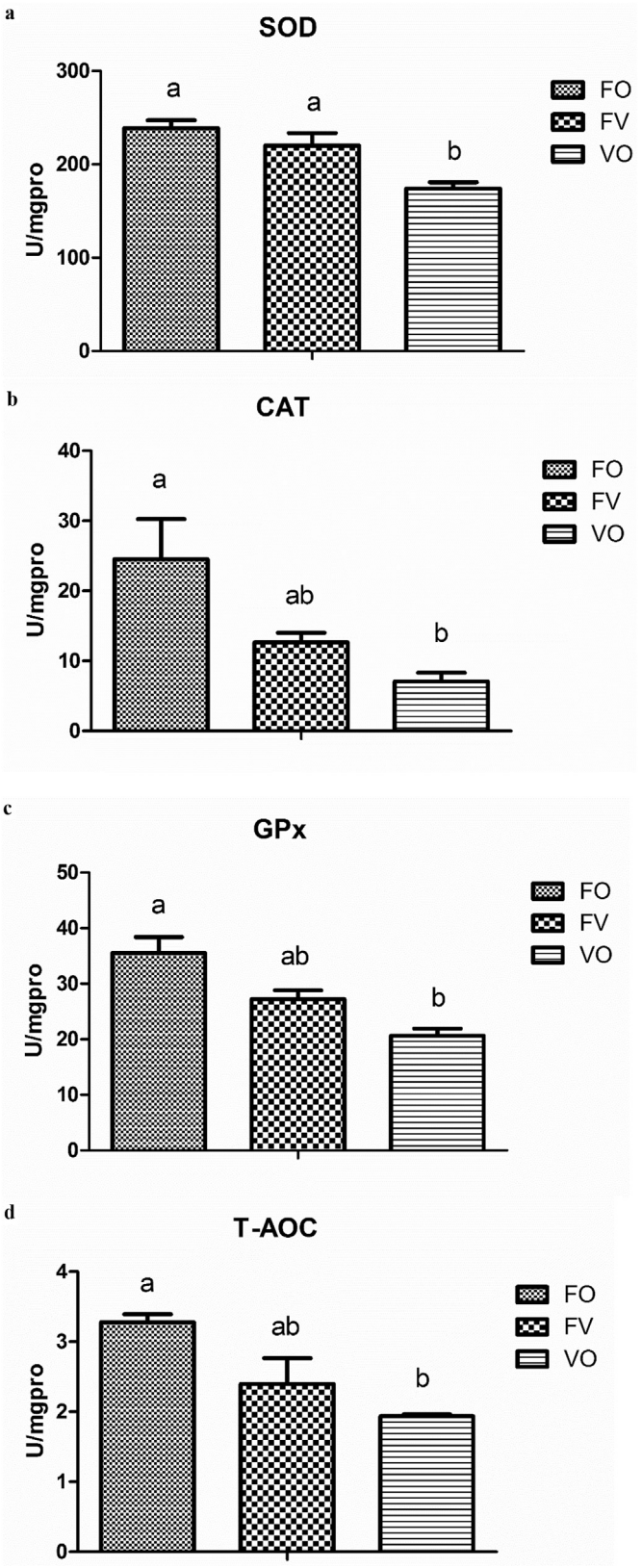


**Fig. 2. VO decrease liver antioxidant enzyme activities in Japanese sea bass.** Enzyme activities are expressed as U per milligram protein. SOD: superoxide dismutase, CAT: catalase, GPx: glutathion peroxidase, T-AOC total antioxidant capacity. FO: control diet, with 0% vegetable oil inclusion; FV: diet with 50% vegetable oil and 50% fish oil; VO: dietary fish oil totally replaced by vegetable oil. Values are means ± S.E.M of three replicates. Different letters in each figure presents significant difference among dietary treatments by Tukey's test ($P < 0.05$). S.E.M.: stand error of means.

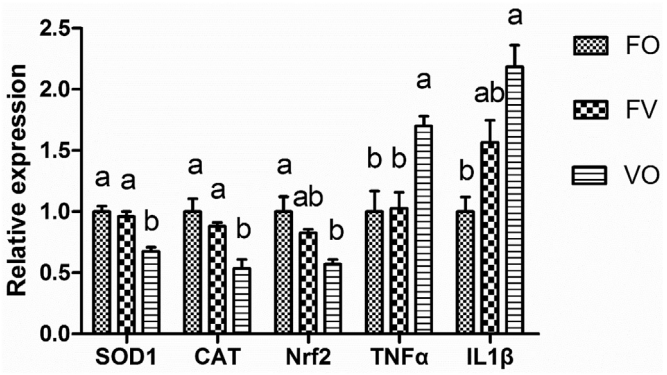


**Fig. 3. VO decreased antioxidant-related genes expression but increased pro-inflammatory cytokine genes expression in Japanese sea bass**. Nrf2: nuclear factor erythroid 2-related factor 2, TNFα: tumor necrosis factor α, IL1β: interleukin1β. FO: control diet, with 0% vegetable oil inclusion; FV: diet with 50% vegetable oil and 50% fish oil; VO: dietary fish oil totally replaced by vegetable oil. Values are means ± S.E.M of three replicates. Different letters in each figure presents significant difference among dietary treatments by Tukey's test ($P < 0.05$). S.E.M.: stand error of means.

## 4. Discussion

Proper nutrition is essential to achieve optimal growth rate and to maintain fish health [11]. In this study, the replacement of dietary fish oil by vegetable oil significantly decreased the specific growth rate (SGR), feed efficiency rate (FER), feed intake (FI) and survival rate (SR) ($P < 0.05$), although the survival rate showed no significant difference between the FV and VO groups [30]. High percentage of dietary VO decreases non-specific immunity in numerous fish species. Data in the present study indicated that the ACP activity significantly decreased in fish fed VO diets. In accordance with this, ACP activity in gilthead sea bream [14] was found significantly decreased when fed the diets with 100% VO inclusion (linseed oil: soybean oil = 1:1). Moreover, a significant increase of CMR was found after the immune challenge in fish fed VO diets. These results indicated that the ACP activity and disease resistance were impaired when dietary FO was totally replaced by VO.

The essential difference between FO and VO is the diversity of fatty acids composition. Changes of dietary fatty acids composition could not only affect non-specific immunity but also alter antioxidant capacity. Investigation in rainbow trout indicated that dietary safflower oil (n-6 PUFA rich) rather than linseed oil (n-3 PUFA rich) significantly increased antioxidant enzyme activities, which was interpreted as a higher oxidation priority of n-6 PUFA over n-3 PUFA [15]. However, in the present study, the total n-6 PUFA content of the VO diets was higher than that of the FO diets (Table 2), but the activities of liver antioxidant enzymes SOD, CAT, GPx and T-AOC all significantly decreased in fish fed VO diets. Therefore, the conclusion that oxidation has a higher priority of n-6 PUFA, which in turn induced antioxidant response, may not be persuasive to the present results. In contrast, the decrease of dietary n-3 LC-PUFA content (from 3.10 to 0.14 mg/g dry diets [30]) resulted from dietary FO replaced by VO, may account for the impairment of antioxidant capacity. Supportive evidence was reported in large yellow croaker of which the serum SOD activity increased with dietary n-3 LC-PUFA increasing from 0.15% to 0.60% [13]. Coincidentally, liver SOD and CAT enzyme activities significantly increased in Japanese sea bass when fed the diet with moderate (0.56%) level of n-3 LC-PUFA [19]. Moreover, the beneficial effects of FO on antioxidant capacity was found in rabbit and rat [20–22]. These results may be explained as the advantage of n-3 LC-PUFA in activating the Nrf2 signaling pathway. The Nrf2 was integrated into nucleus and bound with antioxidant response element (ARE) region, which activated the translation of type II detoxifying enzyme genes, such as superoxide dismutase (SOD), heme oxygenase 1 (HO1), glutathione peroxidase (GPx) and catalase (CAT) [23]. In this study, the nucleus Nrf2 expression of both the mRNA and protein level significantly decreased in fish fed VO diets. Antioxidant enzyme activities and related genes expression indicated that a total replacement of dietary FO by VO decreased the antioxidant capacity in Japanese sea bass which may be due to the attenuation of Nrf2 signaling pathway.

Nrf2 not only is an important transcription factor responsible for maintaining antioxidant capacity, but also plays a role in attenuating pro-inflammatory response (reviewed by Kim et al. [38]).

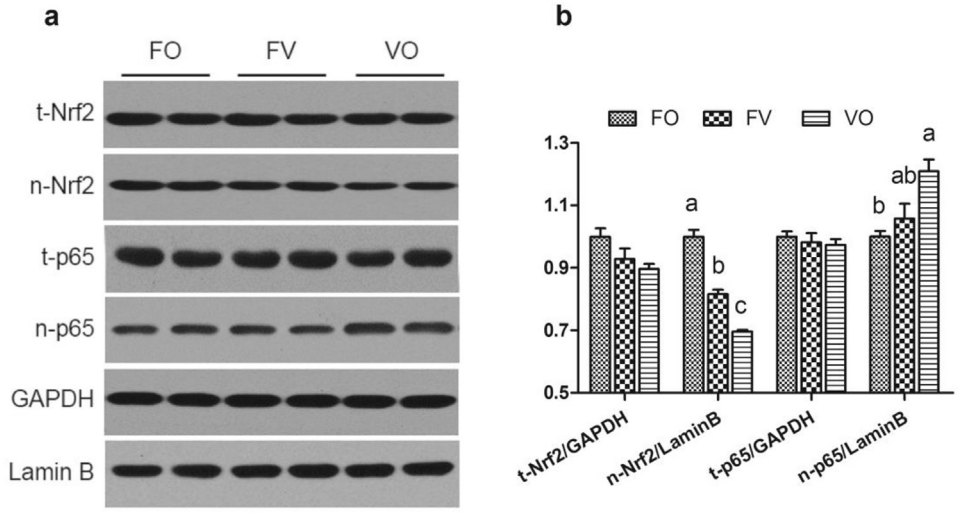


**Fig. 4. VO suppressed Nrf2 signaling but activated NF-κB signaling in liver of Japanese sea bass.** Fig. 4a presents expression levels of Nrf2 and p65 protein in fish feed different diets. Fig. 4b features the ratio of total Nrf2 to GAPDH (*t*-Nrf2/GAPDH), nucleus Nrf2 to Lamin B (*n*-Nrf2/LaminB), total p65 to GAPDH (*t*-p65/GAPDH) and nucleus p65 to Lamin B (*n*-p65/LaminB). GAPDH and Lamin B are selected as total and nucleus reference proteins, respectively. Data are expressed as A.U. of the Western blots and are depicted as a ratio of target protein to the reference protein. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. FO: control diet, with 0% vegetable oil inclusion; FV: diet with 50% vegetable oil and 50% fish oil; VO: dietary fish oil totally replaced by vegetable oil. Values are means ± S.E.M of three replicates. Different letters in each figure presents significant difference among dietary treatments by Tukey's test ($P < 0.05$). S.E.M.: stand error of means.

During the early phase of inflammation, over-expression of Nrf2 inhibits the production of pro-inflammatory cytokines, such as TNFα and IL1β [39,40]. In this study, the mRNA expression of TNFα and IL1β in fish fed FO diets significantly decreased, which may be partly due to the increased expression of Nrf2 of both the mRNA and protein level. Evidence has shown that the NF-κB is responsible for the transcription of the genes encoding many pro-inflammatory cytokines in mammalian [41]. The way Nrf2 plays a role in anti-inflammation may be the cross talk between Nrf2 and NF-κB signaling pathway. Previous study found that NF-κB DNA binding activity and the protein level of the NF-κB subunit p65 were significantly higher in Nrf2 knockout mice than in the wild-type counterpart. Moreover, pro-inflammatory genes were highly expressed upon LPS stimulation in Nrf2 knockout mice [42]. In the present study, dietary VO significantly attenuated Nrf2 expression of both mRNA and nucleus protein level but enhanced the expression of nucleus p65 protein. The potential mechanism may be that FO rather than VO activated the Nrf2 signaling pathway, which in turn attenuated NF-κB mediated pro-inflammatory cytokines production. Although no direct evidence showing that FO exerted anti-inflammatory effects directly through Nrf2, it was indicated that when cells were treated with Nrf2-activating agents, inhibition of NF-κB activity was observed. In EA.hy926 cells, pre-treatment of DHA induced Nrf2 translocation to the nucleus but inhibited TNFα-induced pro-inflammatory response by attenuating NF-κB activation [43]. Likewise, DHA and EPA anti-inflammatory effect in macrophages was partly attenuated in Nrf2 knockout mice when compared with that in the wild-type counterpart [44]. Apart from the Nrf2 signaling pathway, fatty acids could also modulate NF-κB activation directly. For example, EPA was found to decrease the LPS induced activation of NF-κB in human monocytes [45]. DHA attenuated NF-κB activation in macrophages, an effect that involved decreased IκB phosphorylation [46]. Similar results were reported by Oliver et al. in macrophage of mice [47]. Moreover, the decrease of dietary arachidonic acid content result form dietary FO replaced by VO may also account for pro-inflammatory, because arachidonic acid was reported to decreased TNFα content in the serum of grass carp [48]. Therefore, dietary FO may decrease the TNFα and IL1β transcription by activating Nrf2 indirectly or suppressing the NF-κB signaling activation directly, which needs further investigation.

## 5. Conclusion

Dietary VO decreased the ACP activity, disease resistance and liver antioxidant capacity but increased the inflammatory response by activating NF-κB signaling in Japanese sea bass.

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