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Vegetable oil induced inflammatory response by altering TLR-NF-κB signalling, macrophages infiltration and polarization in adipose tissue of large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

High level of vegetable oil (VO) in diets could induce strong inflammatory response, and thus decrease nonspecific immunity and disease resistance in most marine fish species. The present study was conducted to investigate whether dietary VO could exert these anti-immunological effects by altering TLR-NF-κB signalling, macrophages infiltration and polarization in adipose tissue of large yellow croaker (*Larimichthys crocea*). Three iso-nitrogenous and iso-lipid diets with 0% (FO, fish oil, the control), 50% (FV, fish oil and vegetable oil mixed) and 100% (VO, vegetable oil) vegetable oil were fed to fish with three replicates for ten weeks. The results showed that activities of respiratory burst (RB) and alternative complement pathway (ACP), as well as disease resistance after immune challenge were significantly decreased in large yellow croaker fed VO diets compared to FO diets. Inflammatory response of experimental fish was markedly elevated by VO reflected by increase of pro-inflammatory cytokines (IL1β and TNFα) and decrease of anti-inflammatory cytokine (arginase I and IL10) genes expression. TLR-related genes expression, nucleus p65 protein, IKKα/β and IκBα phosphorylation were all significantly increased in the AT of large yellow croaker fed VO diets. Moreover, the expression of macrophage infiltration marker proteins (cluster of differentiation 68 [CD68] and colony-stimulating factor 1 receptor [CSF1R]) was significantly increased while the expression of anti-inflammatory M2 macrophage polarization marker proteins (macrophage mannose receptor 1 [MRC1] and cluster of differentiation 209 [CD209]) was significantly decreased in the AT of large yellow croaker fed VO diets. In conclusion, VO could induce inflammatory responses by activating TLR-NF-κB signalling, increasing macrophage infiltration into adipose tissue and polarization of macrophage in large yellow croaker.

1. Introduction

Fish oil (FO), which contains a relatively high content of long-chain polyunsaturated fatty acids (LC-PUFA), is the traditionally major lipid component of the fish diet. With the development of aqua-feed industry, the increasing demand for FO has post great pressure on fishery resources that have been exploited at their maximum sustainable limit [1]. Vegetable oil (VO), with relatively considerable output, acceptable price, relatively low organic contaminant status, and relatively high content of unsaturated fatty acids, is a promising alternative to FO. However, high inclusion of VO resulted in decreased non-specific immunity parameters, especially for marine fish species, such as gilthead sea bream (*Sparus aurata*) [2,3], European sea bass (*Dicentrarchus labrax*), Atlantic salmon (*Salmo salar*) [4], and large yellow croaker (*Larimichthys crocea*) [5]. Moreover, numerous studies indicated that VO resulted in the overexpression of pro-inflammatory cytokine genes expression and inflammatory response in Senegalese sole

Abbreviations: TLR, toll-like receptor; NF-κB, nuclear factor kappa beta; RB, respiratory burst; LZM, lysozyme; ACP, alternative complement pathway; CMR, cumulative mortality rate; DHA, ducosahexenoic acid; EPA, eicosapentaenoic acid; AT, adipose tissue; IKKα/β, inhibitor of NF-κB kinase α/β; IκBα, inhibitor of NF-κB; CD68, cluster of differentiation 68; CSF1R, colony-stimulating factor 1 receptor; MRC1, macrophage mannose receptor 1; CD209, cluster of differentiation 209; IL1β, interleukin 1β; TNFα, tumour necrosis factor α; Arg I, arginase I; IL10, interleukin 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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(*Solea senegalensis*) [6], gilthead sea bream [4] and large yellow croaker [7]. Persistent attempts have been conducted to elucidate the mechanism of VO in inducing fish immunity problems from the perspective of membrane fluidity, eicosanoid pathways [8], and pattern recognition receptor pathways [9]. Besides, studies have been carried out to reveal the immune-regulation mechanism of VO in the head kidney [4], intestine [4], liver [4], heart [10] because of their close relationship with immunity. However, as far as we know, no information was available about immune response to VO in the adipose tissue (AT) of any fish species.

During the past decade, the immunity role of adipose tissue (AT) has attracted ever-increasing attention in mammal studies since the discovery of inflammatory response induced by macrophages infiltration into AT [11]. AT has been well known to regulate lipid homeostasis by storing excess energy in the form of triglycerides, while more and more studies have indicated that fatty acids are closely related to the accumulation of adipose tissue macrophages (ATMs) [12,13]. The infiltration of ATMs and polarization toward pro-inflammatory M1 type macrophage were confirmed to be closely related to the activation of NF-κB signalling and the over-expression of pro-inflammatory cytokines, such as TNFα, IL6 and IL1β [14]. Mammal studies have verified that the anti-inflammatory role of fish oil was partially resulted from the suppression of ATMs infiltration and decrease of AT pro-inflammatory cytokines expression [15,16]. Besides, the paracrine of pro-inflammatory cytokines by ATMs has been found to induce inflammatory response [17]. The increasing inflammatory response was usually accompanied by the decreasing non-specific immune response in fish species, though the mechanism was still unclear [6,18,19]. In thus, the decrease of non-specific immunity by dietary VO may partially due to the pro-inflammatory cytokines secreted by AT.

To our knowledge, no investigation has been conducted to elucidate the immune regulation mechanisms of VO from the perspective of ATMs accumulation and polarization in AT in this and other fish species. Thus, this study was conducted to investigate non-specific immunity parameters, ATM infiltration and polarization marker proteins expression and TLR-NF-κB signalling in large yellow croaker (*Larimichthys crocea*) in response to dietary VO. It was aimed to better understand the mechanism about how dietary VO induce inflammatory response and decrease fish immunity.

2. Materials and methods

2.1. Animals, diets formulation and animal husbandry

Disease-free and equal sizes of large yellow croaker was from a commercial farm in Ningbo, China. Before the experiment, fish was acclimatized by feeding a control diet for two weeks. Diet formulations and animal husbandry were described in a previous study [20]. Briefly, soybean meal and defatted fish meal were the main protein sources. Three iso-nitrogenous (41% crude protein) and iso-lipid (12% crude lipid) diets were formulated with the replacement of fish oil by vegetable oil as follows: 0% replacement (FO), 50% replacement (FV, fish oil: soybean oil: linseed oil = 2:1:1) and 100% replacement (VO, soybean oil: linseed oil = 1:1). The approximate compositions were analysed and are shown in Table 1. The content of different fatty acids in the experimental diets (mg/g) were determined and are shown in Table 2.

Animal experiment for the large yellow croaker was processed in a net cage system at Xihu Harbor (Ningbo, China). After fasting for 24 h, large yellow croaker (mean weight 8.93 g ± 0.21 g) was randomly divided into 9 floating cages with 60 fish per cage. Each type of diet was randomly divided into 3 parts, and each diet was randomly assigned to a net cage. Fish was fed twice a day to apparent satiation for 70 days. Husbandry was 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 the Ocean University of China.

Table 1
Formulation of the experimental diets (% dry matter) [20].

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
Attractant[h]	0.3	0.3	0.3
Mould 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 of vegetable oil (linseed oil/soybean oil = 1:1) replacing fish oil at 50%.
[c] VO: blend of vegetable oil replacing fish oil at 100%.
[d] Defatted fish meal: 72.1% crude protein and 1.4% crude lipid; white fish meal was defatted with ethanol (fish meal:ethanol = 1:2 (w:v)) at 37°Cfor three replications.
[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 cellulose 16.47 g.
[h] Phagostimulant: Glycine/Betaine = 1:3.
[i] Preservative: Fumarate/Calcium pnpionabe = 1:1.

Table 2
The content of different fatty acids in the experimental diets (mg/g)[a] [20].

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] 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.

2.2. Sample collections

At the end of the feeding trial and after being fasted for 24 h, 5 fish per cage were randomly collected and anaesthetized (MS222; Sigma, USA). Blood samples were collected from the caudal vasculature using a 1-mL syringe, injected into an EP tube and allowed to clot at room temperature for 2 h before being stored for 6 h at 4 °C. The clot was removed, and residual blood cells were separated from the straw-coloured serum by centrifugation (3000 × g, 10 min, 4 °C). The bloodless fish were sacrificed and packed on ice. After removed the abdominal membrane with scalpel, the adipose tissue was scrape from the abdominal wall. Washed with PBS, the adipose tissue was collected in 1.5-mL cryogenic micro-tubes (Sangon, China). All samples were immediately frozen in liquid nitrogen and stored at −80 °C prior to analysis.

2.3. Head kidney macrophage separations and respiratory burst activity assays

Head kidney macrophages were separated as described in a previous study with some modifications [21]. Five fish was randomly collected and dissected to obtain the head kidneys after anaesthetized (MS222; Sigma, USA). Head kidneys, cut into small fragments then washed with L-15 medium (Sigma, USA), were forced to pass through 100 μm cell strainer (Falcon, USA) using 2 mL syringe piston into 50 mL centrifuge tube (Corning, USA). The L-15 medium was supplied with 100 U penicillin and streptomycin, 2 mM L-alanyl-L-glutamine (Thermo Fisher Scientific, USA) and 2% fetal bovine serum (Gibco, USA). Separated cell density was countered in a haemocytometer and modulated to 1×10^7mL. Viability of cells were determined by the trypan blue staining method and was guaranteed >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 [21]. A suspension of head kidney macrophages (100 μL, 1×10^7/mL) was added to a 96-well cell culture plate and centrifuged for 10 min (1500 × g, Sorvall Legend RT, Germany). The supernatant was then replaced by 200 μL of L-15 medium (NBT, Sigma, USA, 1 mg/mL; phorbol 12-myristate 13-acetate, PMA, Sigma, USA, 1 μg/mL). Cell fixation was performed after incubation (40 min, 18 °C, in the dark) using 200 μL of absolute methanol per well. Subsequently, each well was washed with 70% methanol aqueous solution and incubated for 10 min. The procedure was repeated, and the plate was air-dried. Blue precipitation formed in the well and was dissolved with 120 μL of a potassium hydroxide aqueous solution (2 M) and DMSO (Sinopharm Chemical Reagent, China). The respiratory burst activity was expressed as the absorbance value detected under a 630-nm wave length.

2.4. Serum lysozyme activity assays

Serum lysozyme activity was measured by the self-contrasted method described in a previous study [22]. Briefly, a reaction mixture of 10 μL of serum and a 1.4 μL 0.2 mg/mL *Micrococcus lysodeikticus* (Sigma, USA) suspension was incubated at 25°Cfor 10 min. The absorbance value was detected under a 540-nm wave length once per minute. One unit was defined as the absorbance value that attenuated 0.001 in one minute utilizing 1 mL serum.

2.5. Alternative complement pathway (ACP) activity

The alternative complement pathway activity was determined as previously described [23]. Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 mL was dispensed into test tubes, and the total volume was brought up to 0.25 mL with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetra acetic acid (EGTA) and Mg^{2+}. Subsequently, 0.1 mL of rabbit red blood cells (RaRBC) was added to each tube. After incubation for 90 min at 20 °C, 3.15 mL of 0.9% NaCl was added to the test tubes. Samples were centrifuged at 1600 × g for 5 min at 4 °C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at 414 nm. A lysis curve was prepared to determine the volume of serum that yielded 50% hemolysis, and the value of ACH50 units/mL was obtained for each group.

2.6. Mortality after challenged with Vibrio anguillarum

At the end of the feeding trial, large yellow croaker was immune challenged with *Vibrio anguillarum* (provided by Pro. Jing Xing, Ocean University of China). Procedures for bacteria preparation were according to methods described previously with some modifications [24]. Briefly, the bacterial strain was streaked onto blood agar plates and grow at room temperature for 20 h. A single colony was chosen for expanding the culture in a liquid medium at 37 °C for 12 h. Just before the immune challenge, the *V. anguillarum* culture was suspended in phosphate-buffer saline. The suspension was kept on ice before use. Before injection, ten fish per net cage were anaesthetized with MS222 (Sigma, USA). A one-half lethal concentration of *V. anguillarum* (8×10^8 CFU) was injected into the enterocoelia of the fish according to preliminary experiments. After the injection, the fish were left to recover in an aerated tank before being returned to their original cages. Mortality was monitored every day for 7 days.

2.7. RNA extractions, cDNA synthesis, and quantitative real-time polymerase chain reaction (q-PCR)

Adipose tissue was ground to powder in liquid nitrogen and added to Trizol reagent (Takara, China). Subsequently, total RNA was extracted following the manufacturer's protocol. To remove genomic DNA, extracted RNA was treated with RNase-Free DNase (Takara, China) in 42°Cfor 2 min. The integrity of RNA was detected by electrophoresis using 1.2% denatured agarose gel. The quantity of RNA was determined by a Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, USA). Total RNA with a 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 by the Primer Script ™ RT reagent Kit (Takara, China) following the manufacturer's instructions.

Real-time polymerase chain reaction was performed as previously described [25]. Three replicate extractions were performed for each sample. The primers were designed following the published sequences (Table 3). To calculate the expression of immune-related genes, the comparative CT method ($2^{-\triangle\triangle CT}$ method) was adopted, and the value stood for the n-fold difference relative to the calibration [26].

2.8. Western blot

The nuclear proteins of adipose tissue were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Membrane proteins of adipose tissue were extracted using the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Total adipose tissue proteins were extracted according to methods previously described [27]. The protein content was quantified using a Bradford Protein Assay Kit (Beyotime Institute of Technology, China). An equal amount of protein (20 μg) was loaded into wells

Table 3
Primers used in this study.

Primer names	Forward primer sequence (5′ to 3′)	Tm (°C)	Fragment(bp)	PCR efficiency(%)
L-TLR1-F/R	TGTGCCACCGTTTGGATA/TTCAGGGCGAACTTGTCG	57	95	99
L-TLR2-F/R	TCTGCTGGTGTCAGAGGTCA/GGTGAATCCGCCATAGGA	57	98	98
L-TLR3-F/R	ACTTAGCCCGTTTGTGGAAG/CCAGGCTTAGTTCACGGAGG	58	159	102
L-TLR7-F/R	ATGCAATGAGCCAAAGTCT/CATGTGAGTCAATCCCTCC	54	185	97
L-TLR9-F/R	AACGGAGGTCACAGGAGG/TAGCACCACTGGACAGCAC	55	133	98
L-TLR13-F/R	CCTCCTGTTTATGGTAGTGTCC/GCTCGTCATGGGTGTTGTAG	56	161	98
L-TLR22-F/R	TATGCGAGCAGGAAGACC/CAGAAACACCAGGATCAGC	54	132	96
L-MyD88-F/R	TACGAAGCGACCAATAACCC/ATCAATCAAAGGCCGAAGAT	57	144	98
L-Arg I-F/R	AACCACCCGCAGGATTACG/AAACTCACTGGCATCACCTCA	58	119	99
L-IL10-F/R	AGTCGGTTACTTTCTGTGGTG/TGTATGACGCAATATGGTCTG	55	144	99
L-IL1β-F/R	CATCTGGAGGCGGTGGAGGA/GGGACAGACCTGAGGGTGGT	57	119	100
L-TNFα-F/R	CGTCGTTCAGAGTCTCCTGC/TGTACCACCCGTGTCCCACT	58	189	99
L-βactin-F/R	GACCTGACAGACTACCTCATG/AGTTGAAGGTGGTCTCGTGGA	58	136	100

TLR: toll-like receptor, MyD88: myeloid differentiation factor 88, Arg I: arginase I, IL10: interleukin 10, IL1β: interleukin 1β, TNFα: tumour necrosis factor α.

and separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins in the gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA), followed by membrane blocking at room temperature for 2 h. In the freezer used for the chromatography experiment, PVDF membranes were incubated with primary antibody overnight. The membranes were then washed five times for 3 min each with Tris buffered saline with Tween™ (TBST) and incubated for 2 h with horseradish peroxide (HRP)-conjugated secondary antibody in the TBST. Immune complexes were visualized using an Electrochemiluminescence (ECL) Kit (Beyotime Institute of Technology, China).

Polyclonal *anti*-Lamin B, *anti*-IKKα/β, *anti*-IκBα and Na^+/K^+-ATPase antibodies were obtained from Santa Cruz Biotechnology (USA), whereas polyclonal antiphospho-IKKα/β, antiphospho-IκBα, and *anti*-p65 antibodies were purchased from Cell Signalling Technology (USA). Anti-CD68, *anti*-CSF1R, and anti-CD209 were obtained from Abcam (England). Polyclonal *anti*-MRC1 was obtained from Sangon (China). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and HRP-conjugated secondary antibodies were obtained from Golden Bridge Biotechnology (China).

2.9. Calculations and statistical analysis

Cumulative mortality rate = (Ni −Nf) × 100/Ni. Ni is the initial number of fish in each cage before the immune challenge, while Nf is the final number of fish in each cage that survived after the immune challenge (Ni = 10).

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

3. Results

3.1. Non-specific immunity parameters and disease resistance

Head kidney macrophages respiratory burst (RB) activity of macrophages was significantly decreased in fish fed VO diets when compared with the control ($P < 0.05$) (Fig. 1a). No significant difference in the serum lysozyme (LZM) activity was observed among groups ($P > 0.05$) (Fig. 1b). A significant decrease in the alternative complement pathway (ACP) activity was observed in large yellow croaker fed FV or VO diets ($P < 0.05$) (Fig. 1c). Moreover, a significant decrease of the disease resistance was found in large yellow croaker fed FV or VO diets, which manifested in a significantly higher cumulative mortality rate (CMR) ($P < 0.05$) (Fig. 1d). However, there was no significant difference of ACP activity and CMR between fish fed FV and VO diets ($P > 0.05$).

3.2. TLR-NF-κB signalling activation in adipose tissue

3.2.1. Q-PCR analyses for TLR-related genes expression

TLR-related genes expression in AT of large yellow croaker indicated that TLR1, TLR3, TLR5, TLR9, TLR22 and MyD88 was all significantly increased when fish was fed with FV or VO diets ($P < 0.05$). The mRNA expression of TLR2 and TLR7 was significantly increased when fish was fed with VO diet. There was no significant difference of TLR2 and TLR7 mRNA expression between fish fed FO and FV ($P > 0.05$) (Fig. 2).

3.2.2. Western blot for NF-κB signalling protein expression in AT of large yellow croaker

IKKα/β, p-IKKα/β, IκBα, p-IκBα, total p65 (t-p65) and nucleus p65 (n-p65) protein expression levels were determined by Western blot. Data indicated that the ratio of p-IKKα/β to IKKα/β, and p-IκBα to IκBα was significantly increased in the AT of large yellow croaker when fish was fed FV or VO diets ($P < 0.05$). Ratio of n-p65 to t-p65 was significantly increased in the AT of large yellow croaker when fish was fed with VO diet ($P < 0.05$). No significant difference was observed among IKKα/β, IκBα and t-p65 in all treatments ($P > 0.05$) (see Fig. 3).

3.3. Macrophage infiltration and polarization in AT

3.3.1. Western blot analyses for macrophage infiltration and polarization marker proteins

Western blot analyses for macrophage infiltration marker proteins —cluster of differentiation 68 (CD68) —indicated that CD68 protein expression level was significantly increased in AT of fish fed VO ($P < 0.05$). Similarity, macrophage infiltration marker protein colony stimulating factor 1 receptor (CSF-1R) protein expression level was significantly increased in AT of fish fed FV or VO diets ($P < 0.05$) (Fig. 4a). Moreover, the expression level of M2-type macrophage marker protein, macrophage mannose receptor 1 (MRC1) was significantly decreased in the AT of large yellow croaker when fed VO diets ($P < 0.05$) (Fig. 4a). Another M2-type macrophage marker protein, cluster of differentiation 209 (CD209) was found significantly increased in AT of fish fed with FV or VO diets ($P < 0.05$) (Fig. 4a).

3.3.2. Q-PCR analyses for M1-and M2-type macrophage-related cytokine genes expression

Q-PCR analyses for anti-inflammatory M1 macrophage marker

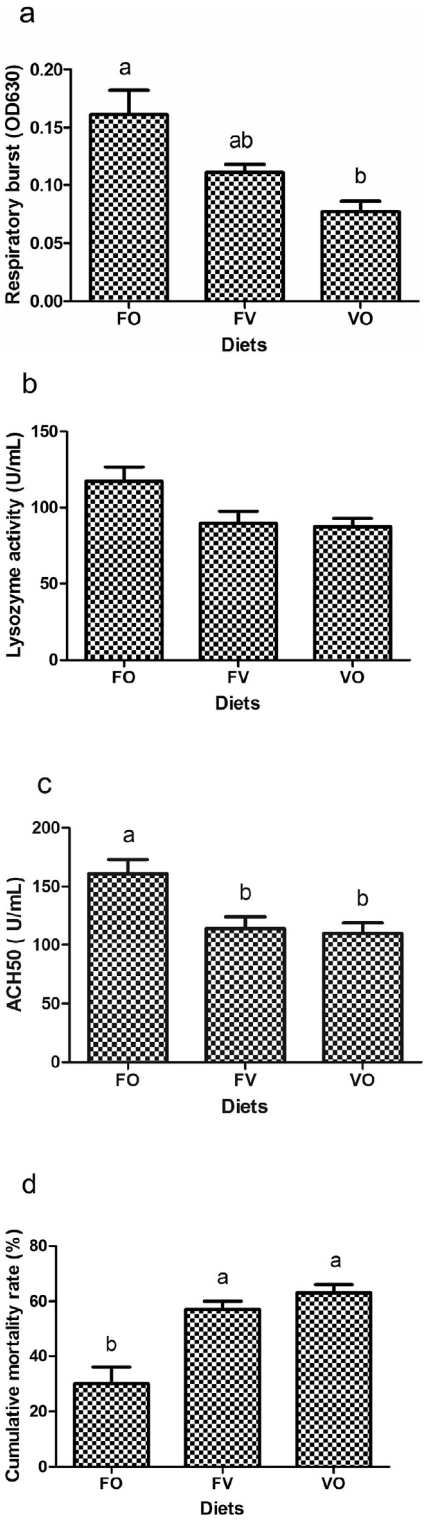

Fig. 1. Non-specific immunity parameters and disease resistance in large yellow croaker. Fig. 1a presents the head kidney macrophages respiratory burst activity. Data is given in absorbance value detected under a 630-nm wave length. Fig. 1b indicates the serum lysozyme activities associated with different dietary treatments. Fig. 1c indicates the alternative complement pathway activity. Data is presented as ACH50. Fig. 1d shows the cumulative mortality rate (CMR) in large yellow croaker after the immune challenge with *V. anguillarum* for 7days. Values are the means ± S.E.M of three replicates. Different letters assigned to the bars in each figure represent significant differences among the dietary treatments using Tukey's test ($P < 0.05$). S.E.M.: standard error of the means.

genes indicated that in the AT of large yellow croaker fed VO diet, the IL1β and TNFα expression levels were significantly higher than those of the control group ($P < 0.05$), while no significant difference was found between FV and VO group ($P > 0.05$) (Fig. 4b). Expression level of M2 macrophage marker gene arginase I (Arg I) was significantly lower in the AT of large yellow croaker fed VO diet ($P < 0.05$). Besides, mRNA expression of Arg I expression level was significantly lower in AT of fish fed VO than fed FV ($P < 0.05$) (Fig. 4b). The mRNA expression level of IL10, another M2 macrophage marker gene, was found significantly lower in AT of fish fed FV or VO diets, but no significant difference was observed between FV and VO groups ($P < 0.05$) (Fig. 4b).

4. Discussion

Nonspecific immunity and disease resistance were both decreased when dietary fish oil was partially or totally replaced by vegetable oil according to the findings of extensive studies on several fish species [4]. In this study, RB activity and ACP activity were both significantly decreased in large yellow croaker fed FV or VO diets. Previous study demonstrated that n-3 LC-PUFA promoted RB activity of macrophages in large yellow croaker [4]. Thus, the decrease of RB in the present study could be partially due to the scarce of n-3 LC-PUFA in vegetable oil. In the present study, content of linoleic acid in diets increased with the increasing portion of vegetable oil (Table 2). High proportion of linoleic acid was confirmed to decreased the non-specific immunity parameters in large yellow croaker [7], grouper (*Epinephelus malabaricus)* [28] and Atlantic Salmon [29]. Therefore, the decrease of RB activity and ACP activity may also due to the high content of linoleic acid in diets.

AT is well known to regulate lipid homeostasis by storing excess energy in the form of triglycerides for an extended period of time. Recently, AT has been found to play an important role during the immunity modulation [12]. Although the specific mechanism has not been completely understood, available evidence indicated that inflammation affected by fatty acids in AT was mainly associated with TLR-NF-κB signalling, macrophage infiltration and polarization [30]. In mammals, toll-like receptor 4 (TLR4) was identified as a receptor of fatty acids and a mediator of pro-inflammatory cytokine production by ATMs. Saturated fatty acids and linoleic acids have been demonstrated to activate TLR4 and then mediate the production of pro-inflammatory cytokines in macrophages through activating several serine kinases, such as IκB kinase [31–33]. In contrast, n-3 PUFA suppressed TLR4-mediated pro-inflammatory cytokine production in macrophages. For example, Lee et al. [34] found that DHA suppressed NF-κB signalling by TLR4 in macrophage. In this study, the expression of TLRs and MyD88 in the AT of large yellow croaker fed a FO diet was significantly lower compared to that fed FV and VO diets. In addition, the elevation ratio of p-IKK/IKK, p-IκB/IκB, and n-p65/t-p65 in the AT of large yellow croaker indicated the activation of NF-κB signalling by replacement of dietary FO with VO. These results indicated that the anti-inflammatory role of FO could be partially accomplished by suppressing the activation of TLRs and downstream signalling in the AT of large yellow croaker. The mechanism by which fatty acids modulated TLRs and subsequent signalling was still not completely elucidated. However, evidence suggested that n-3 LC-PUFA play a role in preventing TLR4 translocation into lipid rafts, an initial event that is involved in TLRs and subsequent NF-κB signalling [35]. Moreover, in a recent study, TLR4 recruitment into lipid rafts was inhibited in rats from fish oil group rather than soybean oil (linoleic acid rich) group [36]. Thus, it is speculated that FO rather than VO disrupts the formation of the lipid raft and then suppresses TLRs-NF-κB signalling in the AT of large yellow croaker.

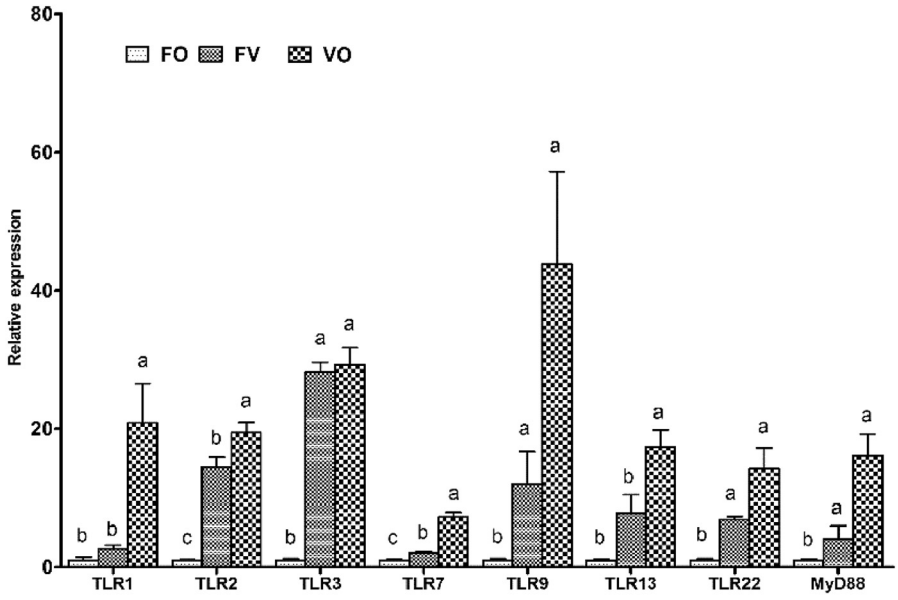

Fig. 2. TLR-related genes expression in AT of large yellow croaker. TLR-related genes expression of TLR1, TLR2, TLR3, TLR7, TLR9, TLR13, TLR22 and MyD88 are determined in the AT of large yellow croaker fed different diets. Values are means ± S.E.M (n = 3). Different letters assigned to the bars represent significant differences using Tukey's test ($P < 0.05$). TLR: toll-like receptor; MyD88: myeloid differentiation factor 88; S.E.M.: stand error of the means.

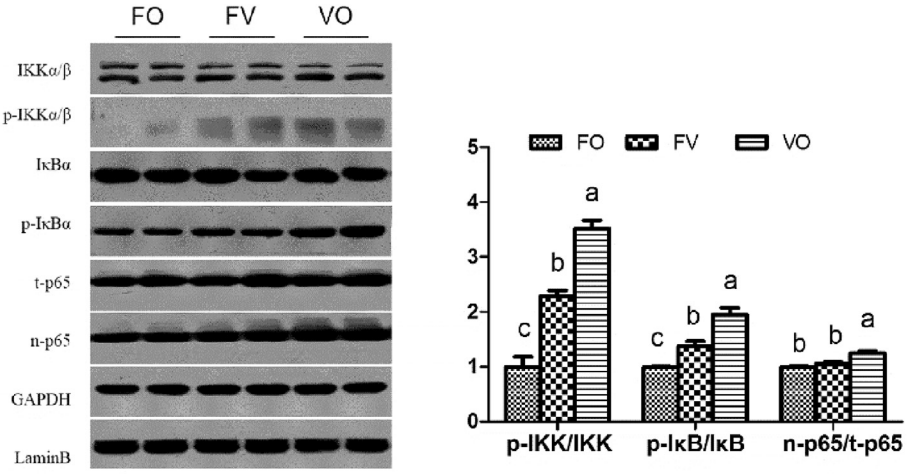

Fig. 3. Western blot analyses for NF-κB signalling activation in the AT of large yellow croaker. The right panel features the ratio of p-IKKα/β to IKKα/β, p-IκB to IκB and n-p65 to t-p65. GAPDH and Lamin B are selected as total and nucleus reference proteins, respectively. Data are expressed as the A.U. of the Western blot and are depicted as a ratio of the target protein to the reference protein. Values are the means ± S.E.M (n = 3). Different letters assigned to the bars represent significant differences using Tukey's test ($P < 0.05$). NF-κB: nuclear factor kappa beta; AT: adipose tissue; IKKα/β: inhibitor of IKKα/β kinase α/β; p-IKKα/β: phosphorylation inhibitor of nuclear factor kappa-B kinase α/β; IκBα: inhibitor of NF-κBα; p-IκBα: phosphorylation inhibitor of NF-κBα; t-p65: total p65; n-p65: nucleus p65; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

In this study, CSF1R and CD68 proteins, two marker proteins for macrophages [14], were both enhanced in the AT of large yellow croaker fed diets with partial or total VO. Notably, the expression of macrophage marker proteins was positively correlated to that of TLR-NF-κB activation in AT of large yellow croaker. This indicated that TLR-NF-κB activation could induce the increased infiltration of macrophages into AT in fish species, just as the process in mammals [4]. Chemokines, such as adipocyte-secreted monocyte chemotactic protein 1 (MCP1), could exert the direct roles in recruiting macrophages into AT [37]. Deletion of the MCP1 gene reduced the accumulation of macrophages while over-expression of the MCP1 gene induced macrophage recruitment into AT [4]. Thus, TLR-NF-κB signalling activation may promote production of chemokines, which induces the infiltration of macrophages into AT [38].

Infiltration of ATMs was usually accompanied by the polarization of macrophages into different types. ATMs phenotypically divided into two types of cells: “classical” pro-inflammatory M1-type macrophages” and “alternatively activated” anti-inflammatory M2-type macrophages. M1-type macrophages significantly contributed to the increased production of pro-inflammatory cytokines, such as TNFα and IL1β. M2-type macrophages were highly active in particle uptake, which was reflected by the expression of non-opsonic pathogen receptors such as MRC1 (or CD206) [39], and CD209 [40]. The M2-type macrophages produced anti-inflammatory cytokines, such as IL10, IL4 and IL13, which was marked by the expression of Arg I and several other genes [34]. Polarization of macrophages was considered to be related to their nutritional status such as dietary lipids, fatty acids

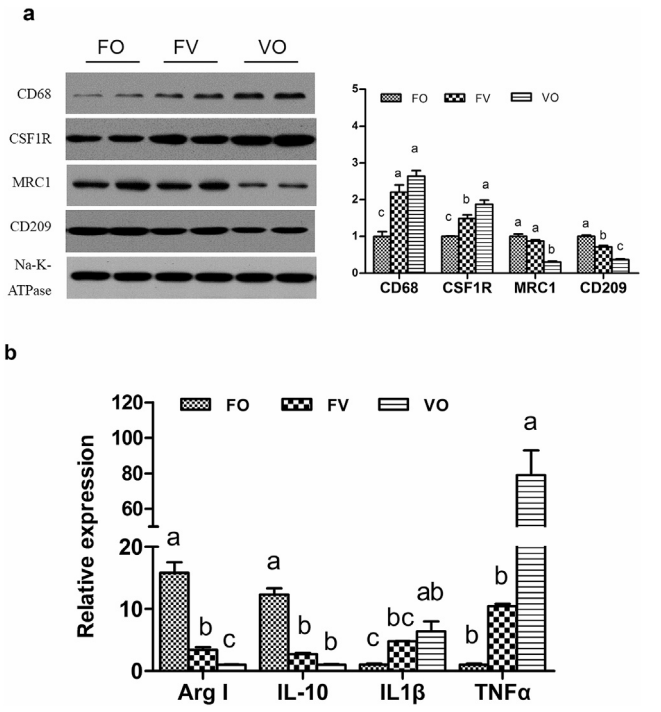

Fig. 4. Analyses of macrophage infiltration and polarization marker proteins and cytokine gene expression in AT. Western blot analyses of macrophage infiltration and proliferation marker proteins, namely, CSF1R, CD68, MRC1 and CD209, in the AT of large yellow croaker when fed FO, FV and VO diets are shown in Fig. 4a. The left panel of Fig. 4a presents the Western blot image of target proteins. The right panel of Fig. 4a presents the ratio of target proteins to Na^+-K^+-ATPase. Q-PCR analyses for cytokine genes (TNFα and IL1β) expressed by M1 macrophage and cytokine genes (Arg I and IL10) expressed by M2 macrophages in the AT of large yellow croaker when fed FO, FV and VO diets are presented in Fig. 4b. Values are means ± S.E.M (n = 3). Different letters assigned to the bars represent significant differences using Tukey's test ($P < 0.05$). CSF1R: colony stimulating factor 1 receptor; CD68: cluster of differentiation 68; MRC1: macrophage mannose receptor 1; CD209: cluster of differentiation 209; IL1β: interleukin 1β; TNFα: tumour necrosis factor α; Arg I: arginase I; IL10: interleukin 10; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

and lipid mediators [4]. In this study, cytokines (TNFα and IL1β) from M1-type macrophages were significantly increased in the AT of large yellow croaker fed FV or VO diets compared to that in FO diets. By contrast, M2-type macrophage marker proteins (MRC1 and CD209) and M2-type macrophage-related cytokines (IL10 and Arg I) were both significantly decreased in the AT of large yellow croaker fed FV or VO diets. This indicated VO increased the polarization of macrophages toward M1 ATMs in AT of large yellow croaker. This finding was in accordant with finding in a rat model, in which mRNA expression of M1 type macrophage infiltration marker gene (F4/80) was significantly higher in rat fed diet with soybean oil inclusion than the control group. Besides, diet with soybean oil inclusion resulted in the over expression of pro-inflammatory cytokines, such as TNFα and IL6, but reduced the expression of anti-inflammatory cytokine IL10 [33]. In contrast, studies indicated that DHA specifically enhanced anti-inflammatory IL-10 secretion and reduced the expression of pro-inflammatory M1 macrophages [41]. The polarization of ATMs was toward M1 ATMs in mice fed a high fat diet, while toward M2 ATMs in obese mice fed diets with n-3 PUFA. Coincidentally, a recent investigation focused on fatty acids in the murine adipocyte macrophage co-culture model and showed that DHA decreased mRNA expression of M1 type ATMs polarization markers while increasing anti-inflammatory cytokines [42]. Thus, it is the relatively higher amount of n-3 LC-PUFA that account for the low polarization rate toward M1 ATMs and low pro-inflammatory response in AT of large yellow croaker fed the FO diet. The relative high content of linoleic acids in FV and VO diets lead to the relative high polarization rate toward M1 ATMs and relative high pro-inflammatory response in AT.

5. Conclusion

Dietary VO decreased the non-specific immunity and disease resistance in large yellow croaker. VO could increase expression of pro-inflammatory cytokine which may results from the activation of TLR-NF-κB signalling, increase of macrophage infiltration into AT and macrophage polarization to M1 type ATMs.

Acknowledgements

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References

[1] M.J. Leaver, J.M. Bautista, B.T. Björnsson, E. Jönsson, G. Krey, D.R. Tocher, et al., Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture, Rev. Fish. Sci. 16 (2008) 73–94.
[2] D. Montero, T. Kalinowski, A. Obach, L. Robaina, L. Tort, M.J. Caballero, et al., Vegetable lipid sources for gilthead seabream (Sparus aurata): effects on fish health, Aquaculture 225 (2003) 353–370.
[3] D. Montero, V. Grasso, M. Izquierdo, R. Ganga, F. Real, L. Tort, et al., Total substitution of fish oil by vegetable oils in gilthead sea bream (Sparus aurata) diets: effects on hepatic Mx expression and some immune parameters, Fish shellfish Immunol. 24 (2008) 147–155.
[4] !!! INVALID CITATION !!!.
[5] R. Zuo, K. Mai, W. Xu, G.M. Turchini, Q. Ai, Dietary ALA, but not LNA, increase growth, reduce inflammatory processes, and increase anti-oxidant capacity in the marine finfish Larimichthys crocea, Lipids 50 (2015) 149–163.
[6] D. Montero, V. Benitez-Dorta, M.J. Caballero, M. Ponce, S. Torrecillas, M. Izquierdo, et al., Dietary vegetable oils: effects on the expression of immune-related genes in Senegalese sole (Solea senegalensis) intestine, Fish shellfish Immunol. 44 (2015) 100–108.
[7] R. Zuo, K. Mai, W. Xu, G.M. Turchini, Q. Ai, Dietary ALA, but not LNA, increase growth, reduce inflammatory processes, and increase anti-oxidant capacity in the marine finfish Larimichthys crocea, Lipids (2014) 1–15.
[8] P.C. Calder, Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance, Biochimica Biophysica Acta (BBA)-Molecular Cell Biol. Lipids 1851 (2015) 469–484.
[9] R. Zuo, Q. Ai, K. Mai, W. Xu, J. Wang, H. Xu, et al., Effects of dietary n-3 highly unsaturated fatty acids on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (Larmichthys crocea) following natural infestation of parasites (Cryptocaryon irritans), Fish shellfish Immunol. 32 (2012) 249–258.
[10] L. Martinez-Rubio, S. Morais, Ø. Evensen, S. Wadsworth, K. Ruohonen, J.L. Vecino, et al., Functional feeds reduce heart inflammation and pathology in Atlantic salmon (Salmo salar L.) following experimental challenge with Atlantic salmon reovirus (ASRV), PloS one 7 (2012) e40266.
[11] H. Xu, G.T. Barnes, Q. Yang, G. Tan, D. Yang, C.J. Chou, et al., Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance, J. Clin. Invistig. 112 (2003) 1821–1830.
[12] C.N. Lumeng, J.L. Bodzin, A.R. Saltiel, Obesity induces a phenotypic switch in adipose tissue macrophage polarization, J. Clin. Invistig. 117 (2007) 175–184.
[13] K.R. Coenen, M.L. Gruen, A. Chait, A.H. Hasty, Diet-induced increases in adiposity, but not plasma lipids, promote macrophage infiltration into white adipose tissue, Diabetes 56 (2007) 564–573.
[14] S.P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R.L. Leibel, A.W. Ferrante, Obesity is associated with macrophage accumulation in adipose tissue, J. Clin. Invistig. 112 (2003) 1796–1808.
[15] J. Todoric, M. Löffler, J. Huber, M. Bilban, M. Reimers, A. Kadl, et al., Adipose

tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n− 3 polyunsaturated fatty acids, Diabetologia 49 (2006) 2109–2119.
[16] V. Saraswathi, L. Gao, J.D. Morrow, A. Chait, K.D. Niswender, A.H. Hasty, Fish oil increases cholesterol storage in white adipose tissue with concomitant decreases in inflammation, hepatic steatosis, and atherosclerosis in mice, J. Nutr. 137 (2007) 1776–1782.
[17] A.A. De Boer, J.M. Monk, L.E. Robinson, Docosahexaenoic acid decreases pro-inflammatory mediators in an in vitro murine adipocyte macrophage co-culture model, PloS one 9 (2014) e85037.
[18] D. Montero, F. Mathlouthi, L. Tort, J.M. Afonso, S. Torrecillas, A. Fernandez-Vaquero, et al., Replacement of dietary fish oil by vegetable oils affects humoral immunity and expression of pro-inflammatory cytokines genes in gilthead sea bream Sparus aurata, Fish shellfish Immunol. 29 (2010) 1073–1081.
[19] S.L. Seierstad, Ø. Haugland, S. Larsen, R. Waagbø, Ø. Evensen, Pro-inflammatory cytokine expression and respiratory burst activity following replacement of fish oil with rapeseed oil in the feed for Atlantic salmon (Salmo salar L.), Aquaculture 289 (2009) 212–218.
[20] X. Dong, Comparision Study on Regulation of Delta 6 Fatty Acyl Desaturase Among Rainboe Trout, Japanese Seabass and Large Yellow Croaker, Ocean University of China, Qingdao: Qingdao Shandong, 2015.
[21] C.J. Secombes, Isolation of salmonid macrophages and analysis of their killing activity, Tech. fish Immunol. 1 (1990) 137–154.
[22] A.E. Ellis, Lysozyme assays, Tech. fish Immunol. 1 (1990) 101–103.
[23] Q. Ai, K. Mai, L. Zhang, B. Tan, W. Zhang, W. Xu, et al., Effects of dietary β-1, 3 glucan on innate immune response of large yellow croaker, Pseudosciaena crocea, Fish Shellfish Immunol. 22 (2007) 394–402.
[24] B. Ching, S. Jamieson, J. Heath, D. Heath, A. Hubberstey, Transcriptional differences between triploid and diploid Chinook salmon (Oncorhynchus tshawytscha) during live Vibrio anguillarum challenge, Heredity 104 (2010) 224–234.
[25] R. Zuo, Q. Ai, K. Mai, W. Xu, J. Wang, H. Xu, et al., Effects of dietary docosahexaenoic to eicosapentaenoic acid ratio (DHA/EPA) on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (Larmichthys crocea) following natural infestation of parasites (Cryptocaryon irritans), Aquaculture 334–337 (2012) 101–109.
[26] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, methods 25 (2001) 402–408.
[27] D. Xu, G. He, K. Mai, H. Zhou, W. Xu, F. Song, Postprandial nutrient-sensing and metabolic responses after partial dietary fishmeal replacement by soybean meal in turbot (Scophthalmus maximus L.), Br. J. Nutr. 115 (2016) 379–388.
[28] F.-C. Wu, H.-Y. Chen, Effects of dietary linolenic acid to linoleic acid ratio on growth, tissue fatty acid profile and immune response of the juvenile grouper Epinephelus malabaricus, Aquaculture 324–325 (2012) 111–117.
[29] I.K. Petropoulos, K.D. Thompson, A. Morgan, J.R. Dick, D.R. Tocher, J. G Bell, Effects of substitution of dietary fish oil with a blend of vegetable oils on liver and peripheral blood leucocyte fatty acid composition, plasma prostaglandin E2and immune parameters in three strains of Atlantic salmon (Salmo salar), Aquac. Nutr. 15 (2009) 596–607.
[30] M. Masoodi, O. Kuda, M. Rossmeisl, P. Flachs, J. Kopecky, Lipid signaling in adipose tissue: connecting inflammation & metabolism, Biochimica Biophysica Acta (BBA)-Molecular Cell Biol. Lipids 1851 (2015) 503–518.
[31] S. Shoelson, J. Lee, M. Yuan, Inflammation and the IKKß/I? B/NF-? B axis in obesity-and diet-induced insulin resistance, Int. J. Obes. Relat. Metabolic Disord. 27 (2003).
[32] H. Shi, M.V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, J.S. Flier, TLR4 links innate immunity and fatty acid–induced insulin resistance, J. Clin. Invistig. 116 (2006) 3015–3025.
[33] X. Wang, Differential effects of high-fat-diet rich in lard oil or soybean oil on osteopontin expression and inflammation of adipose tissue in diet-induced obese rats, Eur. J. Nutr. 52 (2013) 1181–1189.
[34] J.Y. Lee, J. Ye, Z. Gao, H.S. Youn, W.H. Lee, L. Zhao, et al., Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids, J. Biol. Chem. 278 (2003) 37041–37051.
[35] S.W. Wong, M.-J. Kwon, A.M. Choi, H.-P. Kim, K. Nakahira, D.H. Hwang, Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner, J. Biol. Chem. 284 (2009) 27384–27392.
[36] K. Kim, N. Jung, K. Lee, J. Choi, S. Kim, J. Jun, et al., Dietary omega-3 polyunsaturated fatty acids attenuate hepatic ischemia/reperfusion injury in rats by modulating toll-like receptor recruitment into lipid rafts, Clin. Nutr. 32 (2013) 855–862.
[37] J.K. Olson, S.D. Miller, Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs, J. Immunol. 173 (2004) 3916–3924.
[38] T. Suganami, K. Tanimoto-Koyama, J. Nishida, M. Itoh, X. Yuan, S. Mizuarai, et al., Role of the Toll-like receptor 4/NF-κB pathway in saturated fatty acid–induced inflammatory changes in the interaction between adipocytes and macrophages, Arterioscler., thromb., Vasc. Biol. 27 (2007) 84–91.
[39] M. Stein, S. Keshav, N. Harris, S. Gordon, Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation, J. Exp. Med. 176 (1992) 287–292.
[40] T.B. Geijtenbeek, R. Torensma, S.J. van Vliet, G.C. van Duijnhoven, G.J. Adema, Y. van Kooyk, et al., Identification of DC-SIGN, a novel dendritic cell–specific ICAM-3 receptor that supports primary immune responses, Cell 100 (2000) 575–585.
[41] E. Oliver, F.C. McGillicuddy, K.A. Harford, C.M. Reynolds, C.M. Phillips, J.F. Ferguson, et al., Docosahexaenoic acid attenuates macrophage-induced inflammation and improves insulin sensitivity in adipocytes-specific differential effects between LC n-3 PUFA, J. Nutr. Biochem. 23 (2012) 1192–1200.
[42] A.A. De Boer, J.M. Monk, L.E. Robinson, Docosahexaenoic acid decreases pro-inflammatory mediators in an in vitro murine adipocyte macrophage co-culture model, PloS one 9 (2014) e85037.