Comparative Biochemistry and Physiology, Part B 201 (2016) 21–28

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Molecular cloning and functional characterization of arachidonate 5-lipoxygenase (Alox5), and its expression in response to the ratio of linolenic acid to linoleic acid in diets of large yellow croaker (*Larmichthys crocea*)

Tianjiao Wang, Rantao Zuo, Kangsen Mai, Wei Xu, Qinghui Ai *

[a] The Key Laboratory of Aquaculture Nutrition and Feed (Ministry of Agriculture), Ocean University of China, Qingdao, Shandong 266003, People's Republic of China
[b] Key Laboratory of Mariculture (Ministry of Education of China), Ocean University of China, Qingdao, Shandong 266003, People's Republic of China

ARTICLE INFO

Article history:
Received 23 April 2016
Received in revised form 22 June 2016
Accepted 27 June 2016
Available online 1 July 2016

Keywords:
Arachidonate 5-lipoxygenase
Cloning
Prokaryotic expression
Linolenic acid
Linoleic acid
Larmichthys crocea

ABSTRACT

This study was conducted to clone and functionally characterize a full-length cDNA encoding arachidonate 5-lipoxygenase (Alox5) from large yellow croaker (*Larmichthys crocea*) and investigate its gene expression in response to graded dietary ratio of linolenic acid (ALA) to linoleic acid (LNA) (0.03, 0.06, 0.45, 0.90 and 1.51). An isolated 2372 bp cDNA clone of Alox5 contained an open reading frame spanning 2025 bp encoding a protein with the ability to modify arachidonate acid (AA) to 5-hydroxyeicosatetraenoic (5-HETE). In the liver, the Alox5 mRNA expression levels significantly increased to the maximum when the dietary ALA/LNA increased from 0.03 to 0.06, and then significantly decreased with dietary ALA/LNA increased to 1.51 ($P < 0.05$). In the kidney, the expression levels of Alox5 of fish fed diets with low dietary ALA/LNA (0.03–0.06) were significantly higher than those of fish fed diets with high dietary ALA/LNA (0.45–1.51) ($P < 0.05$). The dual-luciferase reporter assays showed that the nuclear factor kappa B (NF-κB) could act on cognate *cis*-acting elements in the promoter of Alox5 and increased the transcription of Alox5. Results of the present study suggested that the expression of Alox5 is higher in croakers fed high concentrations of LNA compared to those fed high concentrations of ALA, which might be regulated by NF-κB and contribute to the inflammation process by catalyzing the dioxygenation of AA.

1. Introduction

In fish, the lipoxygenase gene family (15-lipoxygenase, 12-lipoxygenase and 5-lipoxygenase) is responsible for alteration of arachidonic acid (AA) into bioactive lipid mediators often associated with inflammation. The most highly studied lipoxygenase is 5-lipoxygenase (Alox5), as it modifies AA into hydroperoxyderivatives (5-HPETE) and further into 5-hydroxyeicosatetraenoic acid (5-HETE) which undergoes modifications by enzymatic reactions to become leukotriene A4 and further converted to other leukotrienes dependent on the leukotriene synthase activity in the cell type examined (Rowley et al., 1995; Kuhn et al., 2002; Rådmark, 2002; Yoshimoto and Takahashi, 2002). The function of lipoxygenase in fish has been investigated in zebrafish (*Danio rerio*) (Chen et al., 1994; Sun and Funk, 1996; Johnson et al., 1998; Epp et al., 2007; Krieg et al., 2013), ALOX5 is the key enzyme in the biosynthesis of pro-inflammatory leukotrienes (Singh et al., 2013). The cellular activity of Alox5 could be regulated on transcriptional level by dietary AA in tongue sole (*Cynoglossus semilaevis*) (Yuan et al., 2015) and Atlantic salmon (*Salmo salar*) (Martins et al., 2012) and relevant to inflammatory response in gilthead sea bream (Maradonna et al., 2015) and Atlantic salmon (Chuang et al., 2008). One of the major inflammation-related transcription factors is the nuclear transcription factor kappa-B (NF-κB), which can bind promoters of genes that express pro-inflammatory cytokines and inflammatory mediators (Hinz and Scheidereit, 2014). In mammals, Alox5 is a downstream gene of NF-κB (HoSHIKO et al., 1990), but whether its transcription regulated by NF-κB in fish is unknown.

With the rapid development of the aquaculture industry, a paucity of fish oil has become a bottleneck, which gives rise to the inclusion of vegetable oils in marine fish diets to partially replace fish oil. However, replacing fish oil with vegetable oils has shown to increase hepatic lipid deposition in Atlantic salmon (Menoyo et al., 2006; Ruyter et al., 2006; Jordal et al., 2007) and gilthead sea bream (Menoyo et al., 2004; Cruz-Garcia et al., 2011). Furthermore, the lipid contents in muscle and abdominal cavity are shown to be increased by dietary vegetable oils replacement (Menoyo et al., 2006; Cruz-Garcia et al., 2011; Torstensen et al., 2011). Nevertheless, decreased lipid contents have also been observed in these tissues in the fish fed the vegetable oils diets (Bell et al., 2001, 2002; Nanton et al., 2007). This disparity effect

* Corresponding author.
E-mail address: qhai@ouc.edu.cn (Q. Ai).

http://dx.doi.org/10.1016/j.cbpb.2016.06.010
1096-4959/

is closely associated with fatty acid composition in various dietary oils. Compared to fish oil with high n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) such as EPA (20:5n-3) and DHA (22:6n-3), plant oil is basically comprised of alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LNA, 18:2n-6). On account of the same elongase and desaturase shared by ALA and LNA, the dietary ratio of ALA to LNA is of importance to biological metabolism (Zuo et al., 2015). In addition to biological metabolism, ALA and LNA also have different functions in the immune system. Previous studies on fish have suggested that AA, LNA and perhaps other n-6 PUFA enhance inflammatory cell responses (Montero et al., 2010), whereas n-3 PUFA such as ALA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) diminish these responses (Erdal et al., 1991; Sheldon and Blazer, 1991; Waagbø, 1994; Montero et al., 2004). Due to their important roles in immunological mechanisms and disease resistance (Erdal et al., 1991; Kiron et al., 1995; Montero et al., 2004; Yao et al., 2009), fatty acids serve as major moderators of immune related genes, such as Mx protein (Montero et al., 2008), tumor necrosis factor-a (TNF-α) (Montero et al., 2010), interleukin 1β (IL-1β) (Montero et al., 2010), toll-like receptors (TLRs) and myeloid differentiation factor 88 (MyD88) (Zuo et al., 2012). However, limited information is available about the expression pattern of fish Alox5 in response to dietary fatty acids.

The large yellow croaker, *Larmichthys crocea*, is an important marine fish species that has been cultured widely in southeast China. The aim of the present study was to clone and functionally characterize the Alox5 in large yellow croaker, and to explore the expression patterns and transcriptional regulation of *Alox* in response to different dietary ratio of ALA to LNA.

2. Materials and methods

2.1. Ethics statement

The protocols for animal care and handling used in this study were approved by the Institutional Animal Care and Use Committee of Ocean University of China (Permit Number: 20001001).

2.2. Animals

The feeding experiment was conducted in Xihu Bay, Ningbo, China, as described thoroughly by Zuo et al. (2015). Specifically, whitefish meal and soybean meal were chosen as the main protein sources. Five isoproteic (42% crude protein) and isolipidic (13% crude lipid) diets were formulated to contain graded ratios of ALA to LNA (0.03,0.06,0.45,0.90 and 1.51) by adding different amounts of sunflower oil, linseed oil and palm oil, and the ratio of 0.03 group was treated as the control group (Tables 1 and 2). Juvenile large yellow croakers were hand-fed to apparent satiation twice a day with different experiment diets for 58 days after two-week temporary rearing to acclimate to the experimental diet and conditions.

2.3. Sample collection

At the termination of the experiment, fish were fasted for 24 h and anesthetized with eugenol (1:10,000) (purity 99%, Shanghai Reagent, China) before sampling. Issues, such as kidney, intestine, spleen, heart, liver, brain and muscle from experimental fish were collected, frozen in liquid nitrogen and then stored at −80 °C for the analysis of immune related genes expression.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples with Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The integrity of isolated RNA was evaluated by visualization on a 1.2% denaturing agarose gel, and its purity and concentration were measured by Nano Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). First-strand cDNA was reverse transcribed from the DNase-treated RNA using PrimeScript ™ RT reagent Kit (Takara, Japan) and 3′, 5′ RACE cDNA were synthesized using SMART™ RACE cDNA Amplification Kit (Clontech, California, USA) according to the reagent's instructions.

2.5. The cloning of full-length Alox5 cDNA

The cloning has been previously described in Dong et al. (2015) with slight modification. Degenerate primers were designed based on highly conserved regions from the genes of other fish to amplify internal fragments, and gene-specific primers were designed based on the known sequences of the internal fragments cDNA to clone the 3′- and 5′-end by rapid amplification of cDNA ends (RACE) through a two-round PCR using the SMARTer™ RACE cDNA Amplification Kit (Clontech, California, USA) (Table 2). PCR amplifications using the primers (Table 3) and Taq DNA Polymerase (Takara, Japan) were performed with an initial denaturation at 95 °C for 3 min and 35 cycles of "95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min", followed by a final extension at 72 °C for 10 min. All PCR products were run on a 1.5% agarose gel, and then purified by SanPrep PCR urification Kit (Sangon

Table 1
Formulation and proximate composition of the experimental diets.

Ingredients	Diet ratio of ALA/LNA				
	0.03	0.06	0.45	0.90	1.51
Sunflower oil[a]	7	1	2	0	0
Palm oil[b]	0	6	3	3	0
Linseed oil[c]	0	0	2	4	7
Other Ingredients[d]	93	93	93	93	93
Crude protein (%)	42.41	42.17	42.01	42.32	42.35
Crude lipid (%)	13.39	12.98	13.04	13.87	14.55
ALA/LNA	0.03	0.06	0.45	0.9	1.51

[a] Sunflower oil:Palmitic acid (16:0) content, 7.56% TFA; Oleic acid (18:1n-9) content, 12.89% TFA; Linoleic acid (18:2n-6) content: 58.85% TFA; Linolenic acid (18:3n-3) content: 0.08% TFA, which was bought from Liqun supermarket, Qingdao, China.
[b] Palm oil: Palmitic acid (16:0) content, 34.44% TFA; Oleic acid (18:1n-9) content, 47.31% TFA; Linoleic acid (18:2n-6) content: 12.56% TFA; Linolenic acid (18:3n-3) content: 0.24% TFA, which was bought from Liqun supermarket, Qingdao, China.
[c] Linseed oil:Palmitic acid (16:0) content, 5.25% TFA; Oleic acid (18:1n-9) content, 19.40% TFA; Linoleic acid (18:2n-6) content: 13.52% TFA; Linolenic acid (18:3n-3) content: 47.53% TFA, which was bought from Liqun supermarket, Qingdao, China.
[d] Other Ingredients: All treatments contain 30.00% White fish meal, 26.95% Soybean meal, 22.50% Wheat meal, 5.00% Casein, 2.00% Mineral premix, 2.00% Vitamin premix, 0.40% Attractant, 0.10% Mold inhibitor, 0.05% Yttrium oxide, 9.00% Fish oil.

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

Fatty acid	Dietary ratio of ALA/LNA[a]				
	0.03	0.06	0.45	0.90	1.51
∑ SFA[b]	20.47	33.29	26.9	26.56	19.59
∑ MUFA[c]	26.37	35.18	25.57	29.02	23.66
∑ n-6 PUFA[d]	43.95	22.84	24.34	19.44	20.06
∑ n-3 PUFA[e]	8.45	8.09	17.12	23.29	35.66
ALA/LNA	0.03	0.06	0.45	0.90	1.51
n-3/n-6 PUFA	0.19	0.35	0.70	1.20	1.78
n-3HUFA[f]	7.02	6.75	6.98	7.33	7.80
ARA/EPA[g]	0.47	0.45	0.45	0.43	0.41
DHA/EPA[h]	0.95	0.93	0.93	0.94	0.92

[a] ALA/LNA: ratio of linonlenic acid to linoleic acid.
[b] SFA: saturated fatty acids.
[c] MUFA: mono-unsaturated fatty acids.
[d] n-6 PUFA: n-6 poly-unsaturated fatty acids.
[e] n-3 PUFA: n-3 poly-unsaturated fatty acids.
[f] n-3 HUFA: n-3 highly-unsaturated fatty acids.
[g] ARA/EPA: 20:4n-6/20:5n-3.
[h] DHA/EPA: 22:6n-3/20:5n-3.

Biotech, Shanghai, China). PCR products were cloned into pEASY-T1 simple cloning vector (TransGen, Beijing, China) and sequenced in BioSune (Shanghai, China).

2.6. Alignments of sequences and construction of a phylogenetic tree

The nucleotide and deduced amino acid sequence of Alox5 from large yellow croaker were analyzed using BioEdit 7.0.1. Multiple sequence alignments using the amino acid sequences of Alox5 were made by MegAlign program in DNAStar Package (version 5.01) using the ClustalW method. The phylogenetic tree was constructed based on the complete protein sequences available in Ensembl using the neighbor-joining (N-J) algorithm within MEGA 5.0.

2.7. Quantitative realtime PCR (qRT-PCR) analysis

First-strand cDNA was diluted by four times using sterilized double-distilled water. Quantitative realtime PCR (qRT-PCR) was carried out in a quantitative thermal cycler (Mastercyclerep realplex, Eppendorf, Germany). The amplification was performed in a total volume of 25 μl, containing 12.5 μl of 2× SYBR® Premix Ex Taq™II (Takara, Japan), 9.5 μl of sterilized double-distilled water, 1 μl of each primer (10 μM) and 1 μl of the diluted first strand cDNA product. The real-time qPCR amplification began with 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C. Melting curve (1.85 °C increment/min from 58 °C to 95 °C) was performed after the amplification phase for confirmation. Each sample was run in triplicate. The primers sequence for Real-time RT-PCR were designed based on the full-length of Alox5 cDNA (Table 3). Reference Beta-actin gene (Yao et al., 2009) was used as internal control. The primer amplification efficiency was analyzed according to the following equation $E = 10^{(-1/\mathrm{Slope})-1}$. To calculate the expression of Alox5, the comparative CT method ($2^{-\Delta\Delta t}$ method) was used as described by Livak and Schmittgen (2001).

2.8. Plasmid constructs

For prokaryotic expression, the coding region of the Alox5 cDNA was amplified and subcloned into the *Nde* I/*Eco*RI site of pET16b vectors (Invitrogen, USA) to construct the expression plasmid. For dual-luciferase reporter assays, NF-κB p65 ORF (GenBank Accession No. XM_010731731.1) fragment were amplified, and subcloned into the *Eco*RI/Xhol site of pCS2 + vectors (Invitrogen, USA) to construct pCS-NF-κB expression plasmid; the Alox5 promoter (a 2000 bp fragment, GenBank Accession No. NW_011322448.1) inserted into the *Kpn*I/Xhol site of promoterless pGL3-basic vector (Promega, USA) to construct pGL3- Alox5 reporter plasmids. Plasmids for transfection were prepared using EndoFree Plasmid Mini Kit (OMEGA, USA) according to the manufacturer's instruction.

Table 3
Sequences of the primers used in this study.

Primers	Sequence(5′-3′)
For clone	
Alox5-F	KCCTGGCTTTCCBATGAGTAT
Alox5-R	ATAGAGAAGCCAGTGAAGGA
Alox5–3'F1	CGAGTTTCCCAAGTCCCT
Alox5–3'F2	TGTTCCTGGATCCCCAATGC
Alox5–5'R1	AACTGGTTCACGAACAGGTTCTCTATTGC
Alox5–5'R2	ACTGTCAAACTGGATGTCCCGTGGCA
Universal Primer A Mix (UPM)	CTAATACGACTCACTATAGGGCAAGCAGTG GTATCAACGCAGAGT
Nest Universal Primer (NUP)	CTAATACGACTCACTATAGGGC
For qPCR	
Alox5-RT-F	CAACCCAGTAGTAACCCGAAAGTG
Alox5-RT-R	GTCTGTGCTGTTAGGAGTGATGC
β-actin-F	TTATGAAGGCTATGCCCTGCC
β-actin-R	TGAAGGAGTAGCCACGCTCTGT
For construction of plasmids	
pCS-p65-*Eco*RI	CGGAATTCATGGCGGATGTGT
pCS-p65-Xhol	CCGCTCGAGTCATACGGACG
pGL3- Alox5-*Kpn*I	CGGGGTACCCTGGAACTACTCTGTGG
pGL3- Alox5-Xhol	CCGCTCGAGGGTGGTCACACAAG
BL21- Alox5-*Nde* I	GGGTTTCATATGATGCCATCCTACAC
BL21- Alox5-EcoR I	CCGGAATTCTCAAACAGCCACACT

K = G/T, B = C/G/T.

2.9. Cell culture and transfection

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C in a humidified incubator under 5% CO_2. For DNA transfection, cells were seeded until they were 90–95% confluent at the time of transfection, and then plasmids were transfected by using the Lipofectamine™ 2000 Reagent (Invitrogen, USA) according to the manufacturer's recommendation. For Dual-luciferase reporter assays, 300 ng expression plasmid, 100 ng reporter gene plasmid and 10 ng pRL-CMV renilla luciferase plasmid, and 1 μl Lipofectamine™ 2000 were co-transfected cells in each well in a 24-well plate. All assays were performed with three independent transfections.

2.10. Dual-luciferase reporter assays

Firefly and renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instruction. Briefly, at 24 h post-transfection, HEK293 cells in 24-well plates were washed with 100 ml PBS twice, then lysed with 30 μl 1 × passive lysis buffer at room temperature for 10 min. Cell lysate (20 μl) was transferred to a plate and 50 μl luciferase assay reagent II and 1 × stop & glo reagent were added in sequence, then firefly and renilla luciferase activities were measured, respectively.

2.11. Prokaryotic expression and purification of Alox5

Competent *Escherichia coli* BL21 (DE3) cells were transformed with the pET16b- Alox5 plasmid and grew at 37 °C in LB medium with 100 μg/ml ampicillin and chloramphenicol. The expression of the recombinant enzyme was induced by adding 1 mM (final concentration) IPTG to the main culture and afterwards the culture was incubated over night at 25 °C and 250 rpm. After incubation for 22 h, bacteria were harvested by centrifugation, and resuspended in 5 ml lysis buffer, and lysed by sonication (Branson Ultraschall, Fürth, Germany). After centrifugation at 10,000 g for 15 min, the supernatant was applied to a Ni-agarose column (Merck, Darmstadt, Germany) to purify the histidine-tagged Alox5. The column was washed with washing buffer containing 25 mM imidazole. Finally, the his-tag fusion proteins were eluted with elution buffer containing 200 m Mimidazole.

2.12. Fatty acid oxygenase activity assays

For bacterial activity assays, 3 μg of purified Alox5 was diluted in Dulbecco's PBS/1 mM EDTA/1 mM ATP, pH 7.4. Reaction was started with addition of 1 mM CaCl2 and 20 μM AA in a final reaction volume of 1 ml. After 10 min at 27 °C, 1 ml of ice-cold methanol was added to stop the reaction. Products were analyzed by RP-HPLC after solid phase extraction.

2.13. HPLC analysis

Reverse phase-HPLC was carried out on a Nucleodur C18 Gravity column (Marchery-Nagel, Düren, Germany; 250 × 4 mm, 5 μm particle size) coupled with a guard column (8 × 4 mm, 5-μm particle size). A solvent system of methanol/water/acetic acid (85/15/0.1, by volume)

was used at a flow rate of 1 ml/min. Absorbance was recorded at 235 nm.

2.14. Statistical analysis

Software SPSS 16.0 (SPSS Inc.) was used for all statistical evaluations. 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 ± standard error of the mean.

3. Results

3.1. Characterization of the full-length Alox5 from large yellow croaker

The full-length cDNA of Alox5 from large yellow croaker was 2372 bp (GenBank accession no. **KC991030.1**), including a 5′ untranslated terminal region (UTR) of 143 bp, a 3′ UTR of 204 bp, and an open reading frame (ORF) of 2025 bp encoding a polypeptide of 674 amino acid residues with a predicted molecular weight of 78.06 KDa and a theoretical isoelectric point of 5.83.

3.2. Multiple sequence alignment analysis and phylogenetic analysis of Alox5

The BLAST analysis revealed that Alox5 of the large yellow croaker shared high identity with the known Alox5 of teleosts including *Maylandia zebra*, *Neolamprologus brichardi*, *Haplochromis burtoni*, *Oreochromis niloticus*, 90%; *Pundamilia nyererei*, 89%; *Austrofundulus limnaeus*, *Poecilia Mexicana*, 87%; *Cyprinodon variegatus*, 86%. Phylogenetic analysis by the neighbor-joining method showed that the Alox5 from large yellow croaker is clustered with the Alox5 of other fish species rather than mammalian. The Alox5 from animals and from plants clustered to their corresponding subgroup, respectively. The observed relationships within this cluster reflected the taxonomic positions of the species (Figs. 1 and 2).

3.3. Prokaryotic expression of Alox5 from large yellow croaker

It can be seen from the HPLC analysis that the major AA oxygenation product of the Alox5 was 5-HETE, and only small amounts 5-HETE was found (Fig. 3).

3.4. Expression of Alox5 in tissues

The Alox5 transcripts were broadly expressed in all detected tissues including kidney, intestine, spleen, heart, liver, brain and muscle. The highest expression of Alox5 was in the kidney, approximate 144 times higher relative to the expression level of the muscle where the lowest expression for Alox5 of all tissues sampled was found ($P < 0.05$). The second highest expression of Alox5 was in the intestine, which significantly lower relative to the kidney ($P < 0.05$), and approximate 94 times higher relative to the expression level of the muscle ($P < 0.05$). A nearly equal expression level of Alox5 was found in spleen and heart ($P < 0.05$), which are significantly lower than intestine ($P < 0.05$), but significantly higher than stomach, liver, brain and muscle ($P < 0.05$). The expression level of Alox5 was weakly in stomach, liver, brain and muscle, and no significantly differences between them ($P > 0.05$) (Fig. 4).

3.5. Expression profiles of Alox5 in liver and kidney in response to dietary ratio of ALA/LNA

In liver, the Alox5 expression increased to the maximum as dietary ALA/LNA increased from 0.03 to 0.06, and then decreased with increasing dietary ALA/LNA from 0.06 to 1.51 ($P < 0.05$). The mRNA expression levels of Alox5 were increased by about 1.57-fold in the ratio of 0.06 treatments. The Alox5 transcript levels were down-regulated by 0.51-fold, 1.20-fold and 0.31-fold in the ratio of 0.45, 0.90, 1.51 treatments (Fig. 5A). In kidney, Alox5 transcript levels of fish fed diets with low dietary ALA/LNA (0.03–0.06) were significantly higher than those of fish fed diets high ALA/LNA (0.45–1.51) ($P < 0.05$). No significant differences were observed in the expression of Alox5 of fish fed diets with low ALA/LNA (0.03–0.06) ($P > 0.05$) and high ALA/LNA (0.45–1.51) ($P > 0.05$) (Fig. 5B).

3.6. Dual-luciferase reporter assays

The results showed that compared to the empty vector pCS2 +, expression of recombinant pCS-NF-κB resulted in 2.1-fold ($P < 0.05$) increase in pGL3- Alox5 expression. These results indicated that NF-κB could activate the expression of luciferase reporter genes, suggesting that it could act on cognate *cis*-acting elements in the promoter of Alox5 and promote the transcription of Alox5 (Fig. 6).

4. Discussion

The usage of fish oil as dietary lipid source face the problem of limited supply and escalating cost, and the weakened immunity of fish fed plant oil-based diets may counteract the economic benefits. A way to improve the use of plant-based lipids has been found in the regulation of ALA and LNA metabolism, which is important to the nutrition and immunity of farmed fish. The critical role of Alox5, which is seen as one of many bridges between fatty acid nutrition and immunity, makes the study of fish Alox5 indispensable in this research.

In the present study, the full-length cDNA of Alox5 in large yellow croaker was cloned and functionally characterized for the first time. The cDNA of Alox5 gene contained a 5′-UTR of 143 bp, a 3′-UTR of 204 bp and an ORF of 2025 bp encoding a polypeptide with a length of 674 amino acids. Phylogenetic analysis using the neighbor-joining method showed that the Alox5 of large yellow croaker shared high identity to known Alox5 in both marine fish species and freshwater fish species, such as *Oreochromis niloticus* and *Takifuge rubripes*. Overall, the consensus sequences, predicted topology, and sequence homology of the obtained gene were in strong agreement. The reaction products of AA oxygenation product of purified recombinant Alox5 of large yellow croaker was analysed by HPLC, which indicates that the Alox5 has the ability to modify AA to 5-HETE.

In general, high expression of immune genes are found in immune organs and tissues (Yao et al., 2008, 2009; Zuo et al., 2012). In the present study, Alox5 transcript was observed in all examined tissues. The most predominant expression of Alox5 was found in the kidney and intestine, while the weakest expression was found in the muscle, which is similar to other immune related genes, such as MyD88 and TLR9 (Yao et al., 2008, 2009). In the present study, the weak mRNA expression of Alox5 in the liver, which is different from the immune related genes mentioned above, may due to the specific differences between different immune related genes.

Previous studies showed that high inclusion of dietary n-6 fatty acids exert deleterious effects on the health of fish by influencing fatty acid composition of immune cells, altering eicosanoid production and even chronically increasing the basal expression of certain inflammation related genes (Farndale et al., 1999; Montero et al., 2004, 2008, 2010). These deleterious effects of high dietary n-6 fatty acids levels could be eliminated by increasing n-3/n-6 PUFA. It has been proven that ALA is capable of efficiently eliminating deleterious effects of high LNA content by increasing the n-3/n-6 PUFA (Rollin et al., 2003; Berge et al., 2009). Previous studies showed that the high dietary ALA/LNA could lead to a high inflammation level by increasing the transcription of inflammation factors (Zuo et al., 2015). However, no information is available on the regulation of Alox5 in response to dietary ALA/LNA. In the present study, the major metabolic organ liver and the major immune organ

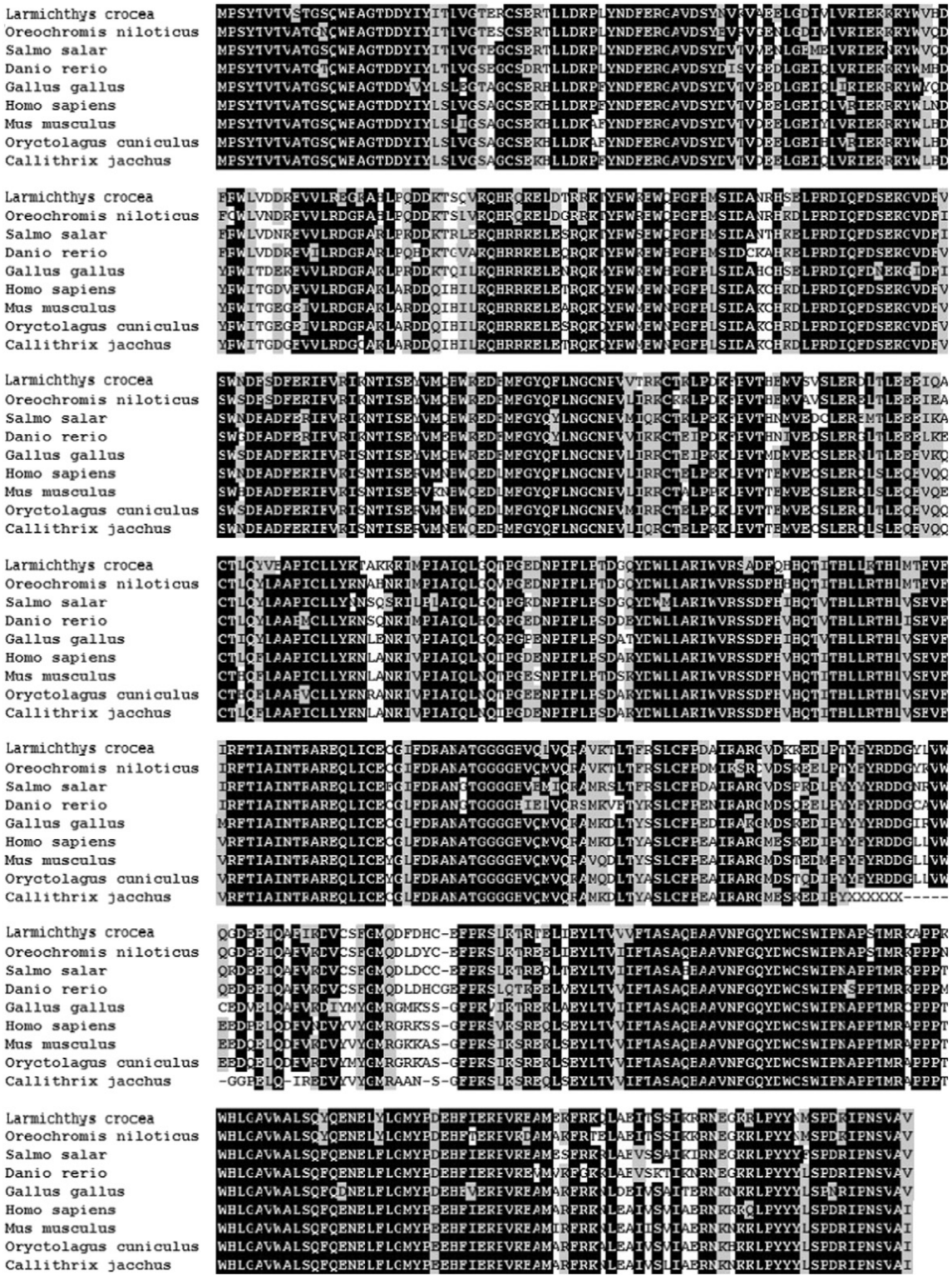

Fig. 1. Multiple alignment of amino acid sequences of Alox5 of *Larmichthy scrocea, Oreochromis niloticus*, *Salmo salar*, *Danio rerio*, *Gallus gallus*, *Homo sapiens*, *Mus musculus*, *Oryctolagus cuniculu* and *Callithrix jacchu.* Alignment was performed using ClustalW2. Identical residues are indicated in black, and similar residues in light gray. Dashes indicate gaps. Identities are shown as black boxes and shaded boxes represent similar amino acids.

kidney were chose to further study. The expression of Alox5 showed a similar changing trend in liver and kidney with a high level of Alox5 expression at the low dietary ALA/LNA (0.03–0.06) and a low level of Alox5 expression at the high dietary ALA/LNA (0.45–1.51). Low dietary ALA/LNA (0.03–0.06) including high dietary n-6 fatty acids induced a higher level of Alox5 expression, which may contribute to the inflammation process by catalyzing the dioxygenation of AA to give the precursor of leukotrienes. The lower expression of Alox5 in high dietary ALA/LNA (0.45–1.51) treatment may due to the anti-inflammatory effects of ALA. Thus, the expression of Alox5 was influenced by dietary ALA/LNA and basically consistent with the inflammatory level.

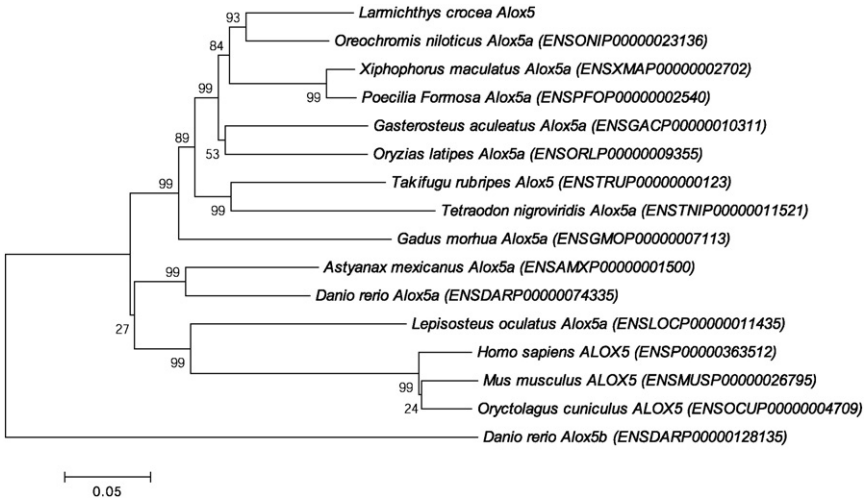

Fig. 2. Phylogenetic tree comparing the deduced amino acid sequence of the newly cloned Alox5 from large yellow croaker with Lox from the other species. The tree was constructed using the neighbor-joining method with MEGA4. The horizontal branch length is proportional to amino acid substitution per site. The numbers represent the frequencies with which the tree topology presented was replicated after 10,000 bootstrap iterations. All accession numbers are from GenBank database.

The qRT-PCR results indicated that dietary ALA/LNA regulated Alox5 expression at the transcriptional level (Fig. 5). The activation of transcription factors NF-κB was studied thoroughly to investigate the induction of Alox5. Dual-luciferase reporter assays showed that pCS-NF-κB activated the luciferase reporter gene pGL3- Alox5 in HEK293 cells. This result indicates that transcription factor NF-κB can act on cognate *cis*-acting elements in the promoter of Alox5 and increase the transcription of Alox5.

In conclusion, the full-length cDNA of Alox5 from the large yellow croaker was successfully cloned and the purified recombinant protein of Alox5 showed the ability to modify AA into 5-HETE. The gene expression of Alox5 in the liver and kidney was stimulated by low dietary ALA/LNA (0.03–0.06) and down-regulated by high dietary ALA /LNA (0.45–1.51), which may be under the regulation of NF-κB and associated with the inflammation level of the experimental fish. Regarding the important role of Alox5 in marine fish nutrition immunity and the relative neglect of its research in the past, further studies, particularly those of the underlying precise biochemical pathways and cellular mechanisms, are needed in the future.

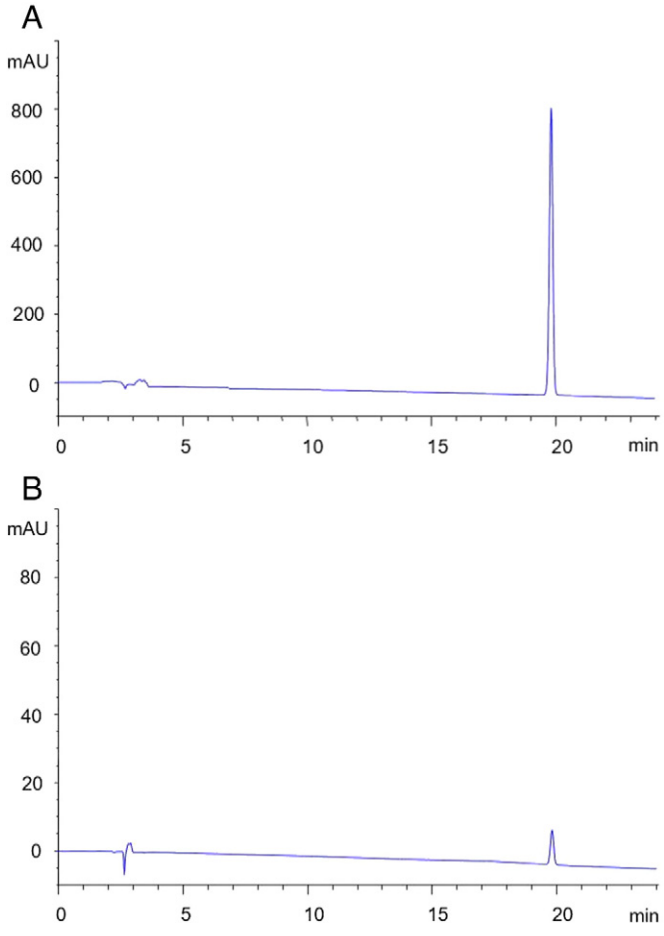

Fig. 3. HPLC product profile of 5-HETE generated from the reaction of large yellow croaker Alox5 with AA as the substrate. A) Standard 5-HETE. B) The reaction products of AA oxygenation product of purified recombinant Alox5 of large yellow croaker. HPLC conditions were as described under experimental procedures. UV absorbance of the eluate was monitored at 235 nm.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This research was supported by National Science Fund for Distinguished Young Scholars of China (31525024), Ph.D. Programs Foundation of Ministry of Education of China (20120132110007) and

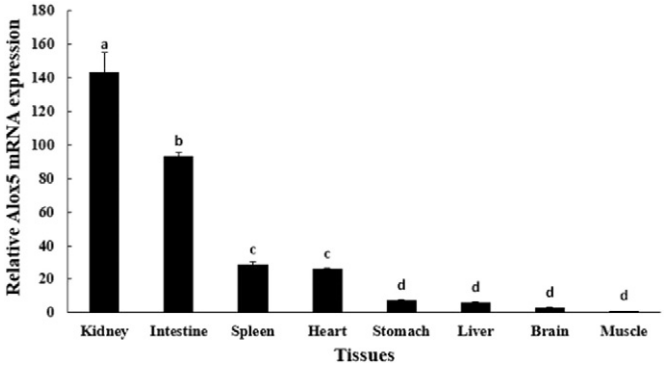

Fig. 4. Real-time PCR result of large yellow croaker Alox5 in different tissues. Relative Alox5 expression was determined by quantitative real-time PCR (qRT-PCR) as described in the Materials and Methods section. Results are expressed as means ± S.E.M. ($n = 3$). Different letters above the bars denote significant differences among tissues at the $P < 0.05$ level ($P = 0.000$) as determined by one-way ANOVA followed by Tukey's test (SPSS).

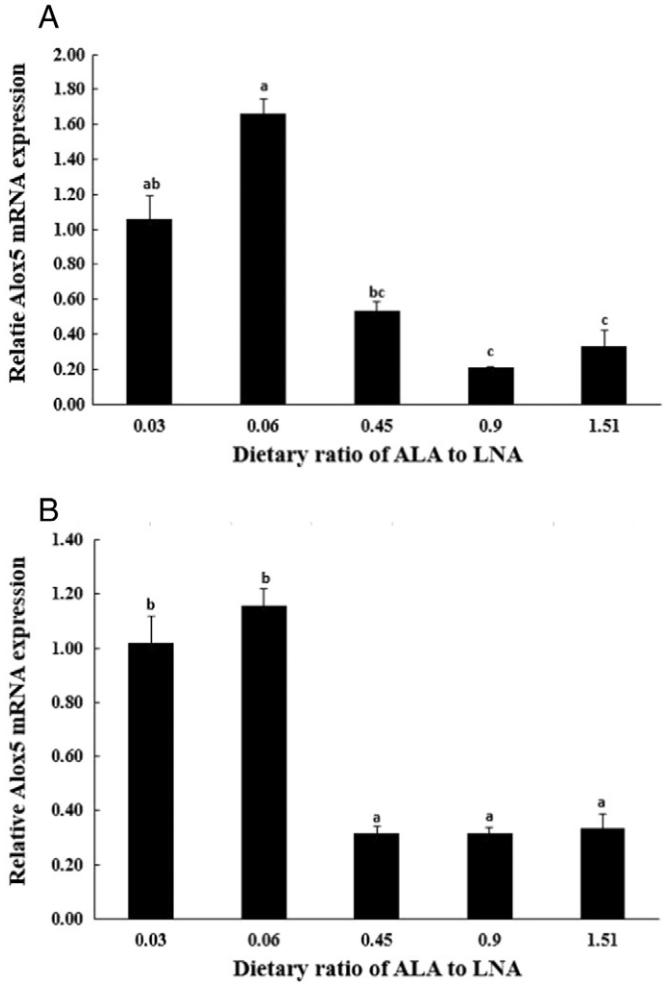

Fig. 5. Relative Alox5 expression levels in (A) liver and (B) kidney of large yellow croaker after fed with different ratio of ALA to LNA in the dietary. Relative Alox5 expression levels were evaluated by quantitative real-time PCR (qRT-PCR) and expressed relative to β-actin levels. Results are expressed as means ± S.E.M. ($n = 3$). Different letters above the bars denote significant differences among dietary groups at the $P < 0.05$ level ($P = 0.000$) as determined by one-way ANOVA followed by Tukey's test (SPSS).

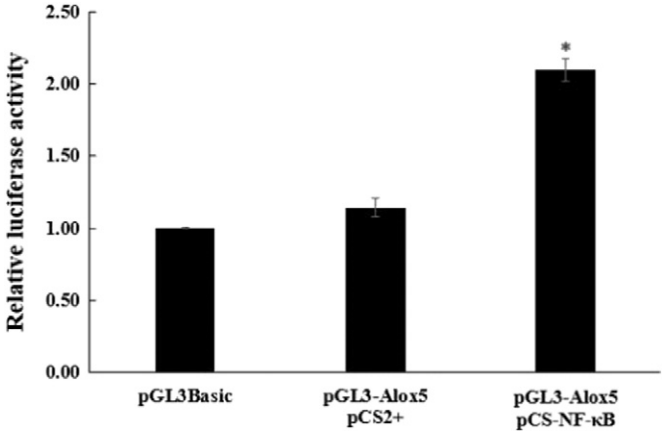

Fig. 6. Relative dual-luciferase activity analysis of NF-κB in the Alox5 promoter in HEK293 cells. The bars indicated relative luciferase activity ($n = 3$). PRL-CMV and pGL3-Basic used as control. The amount relative to the internal control is expressed as mean ± S.E.M. ($n = 3$). $^*P < 0.05$.

National Students' Innovation and Entrepreneurship Training Program (201210423052). We thank W.B. Zhang., Y.J. Zhang and X.J. Dong for their assistance in the study.

T.J. Wang, R.T. Zuo and Q.H. Ai designed the research; T.J. Wang and R.T. Zuo conducted the research; T.J. Wang analyzed the data and wrote the paper; and K.S. Mai and W. Xu contributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

References

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J., Sargent, J.R., 2001. Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism. J. Nutr. 131, 1535–1543.

Bell, J.G., Henderson, R.J., Tocher, D.R., McGhee, F., Dick, J.R., Porter, A., Smullen, R.P., Sargent, J.R., 2002. Substituting Fish Oil with Crude Palm Oil in the Diet of Atlantic Salmon (*Salmo salar*) Affects Muscle Fatty Acid Composition and Hepatic Fatty Acid Metabolism. J. Nutr. 132, 222–230.

Berge, G.M., Witten, P.E., Baeverfjord, G., Vegusdal, A., Wadsworth, S., Ruyter, B., 2009. Diets with different n-6/n-3 fatty acid ratio in diets for juvenile Atlantic salmon, effects on growth, body composition, bone development and eicosanoid production. Aquaculture 296, 299–308.

Chen, X.S., Sheller, J.R., Johnson, E.N., Funk, C.D., 1994. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372, 179–182.

Chuang, W.L., Haugland, Ø., Pan, B.S., Evensen, Ø., 2008. Isoflavone-rich extracts from wooly glycine *Glycine tomentella* inhibits lps-induced tnf-alpha expression in a macrophage cell line of atlantic salmon (*Salmo salar* l.). Mol. Immunol. 45 (15), 3956–3964.

Cruz-Garcia, L., Sanchez-Gurmaches, J., Bouraoui, L., Saera-Vila, A., Perez-Sanchez, J., Gutierrez, J., Navarro, I., 2011. Changes in adipocyte cell size, gene expression of lipid metabolism markers, and lipolytic responses induced by dietary fish oil replacement in gilthead sea bream (*Sparus aurata* L.). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 158, 391–399.

Dong, X., Xu, H., Mai, K., Xu, W., Zhang, Y., Ai, Q., 2015. Cloning and characterization of SREBP-1 and PPAR-α in Japanese seabass *Lateolabrax japonicus*, and their gene expressions in response to different dietary fatty acid profiles. Comp. Biochem. Physiol. B 180, 48–56.

Epp, N., Fürstenberger, G., Müller, K., Juanes, S., Leitges, M., Hausser, I., Thieme, F., Liebisch, G., Schmitz, G., Krieg, P., 2007. 12R-lipoxygenase deficiency disrupts epidermal barrier function. J. Cell Biol. 177, 173–182.

Erdal, J.I., Evensen, Ø., Kaurstad, O.K., Lillehaug, A., Solbakken, R., Thorud, K., 1991. Relationship between diet and immune response in Atlantic salmon (*Salmo salar L.*) after feeding various levels of ascorbic acid and omega-3 fatty acids. Aquaculture 98, 363–379.

Farndale, B.M., Bell, J., Bruce, M.P., Bromage, N.R., Oyen, F., Zanuy, S., Sargent, J.R., 1999. Dietary lipid composition affects blood leucocyte fatty acid compositions and plasma eicosanoid concentrations in European sea bass (*Dicentrarchus labrax*). Aquaculture 179, 335–350.

Hinz, M., Scheidereit, C., 2014. The IκB kinase complex in NF-κB regulation and beyond. EMBO Rep. 15, 46–61.

HoSHIKO, S., Rådmark, O., Samuelsson, B., 1990. Characterization of the human 5-lipoxygenase gene promoter. Proc. Natl. Acad. Sci. U. S. A. 87, 9073–9077.

Johnson, E.N., Brass, L.F., Funk, C.D., 1998. Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc. Natl. Acad. Sci. 95, 3100–3105.

Jordal, A.E.O., Lie, O., Torstensen, B.E., 2007. Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.). Aquac. Nutr. 13, 114–130.

Kiron, V., Fukuda, H., Takeuchi, T., Watanabe, T., 1995. Essential fatty acid nutrition and defence mechanisms in rainbow trout *Oncorhynchus mykiss*. Comp. Biochem. Physiol. A 111, 361–367.

Krieg, P., Rosenberger, S., Juanes, S., Latzko, S., Hou, J., Dick, A., Kloz, U., Hoeven, F., Hausser, I., Esposito, I., Rauh, M., Schneider, H., 2013. Alox3 knockout mice reveal a function of epidermal lipoxygenase-3 as hepoxilin synthase and its pivotal role in barrier formation. J. Invest. Dermatol. 133, 172–180.

Kuhn, H., Walther, M., Kuban, R.J., 2002. Mammalian arachidonate 15-lipoxygenases: Structure, function, and biological implications. Prostaglandins Other Lipid Mediat. 68, 263–290.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Maradonna, F., Nozzi, V., Santangeli, S., Traversi, I., Gallo, P., Fattore, E., et al., 2015. Xenobiotic-contaminated diets affect hepatic lipid metabolism: implications for liver steatosis in *Sparus aurata*, juveniles. Aquat. Toxicol. 167, 257–264.

Martins, D., Rocha, F., Martínez-Rodríguez, G., Bell, G., Morais, S., Castanheira, F., et al., 2012. Teleost fish larvae adapt to dietary arachidonic acid supply through modulation of the expression of lipid metabolism and stress response genes. Br. J. Nutr. 108 (5), 864–874.

Menoyo, D., Izquierdo, M.S., Robaina, L., Gines, R., Lopez-Bote, C.J., Bautista, J.M., 2004. Adaptation of lipid metabolism, tissue composition and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soyabean oils. Br. J. Nutr. 92, 41–52.

Menoyo, D., Diez, A., Lopez-Bote, C.J., Casado, S., Obach, A., Bautista, J.M., 2006. Dietary fat type affects lipid metabolism in Atlantic salmon (*Salmo salar* L.) and differentially regulates glucose transporter GLUT4 expression in muscle. Aquaculture 261, 294–304.

Montero, D., Socorro, J., Tort, L., Caballero, M.J., Robaina, L.E., Vergara, J.M., Izquierdo, M.S., 2004. Glomerulonephritis and immunosuppression associated with dietary essential fatty acid deficiency in gilthead sea bream, *Sparus aurata* L., juveniles. J. Fish Dis. 27, 297–306.

Montero, D., Grasso, V., Izquierdo, M.S., Ganga, R., Real, F., Tort, L., Caballero, M.J., Acosta, F., 2008. 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, 147–155.

Montero, D., Mathlouthi, F., Tort, L., Afonso, J.M., Torrecillas, S., Fernández-Vaquero, A., Negrin, D., Izquierdo, M.S., 2010. 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, 1073–1081.

Nanton, D.A., Vegusdal, A., Rora, A.M.B., Ruyter, B., Baeverfjord, G., Torstensen, B.E., 2007. Muscle lipid storage pattern, composition, and adipocyte distribution in different parts of Atlantic salmon (*Salmo salar*) fed fish oil and vegetable oil. Aquaculture 265, 230–243.

Rådmark, O., 2002. Arachidonate 5-lipoxygenase. Prostaglandins Other Lipid Mediat. 68, 211–234.

Rollin, X., Peng, J.L., Pham, D., Ackman, R.G., Larondelle, Y., 2003. The effects of dietary lipid and strain difference on polyunsaturated fatty acid composition and conversion in anadromous and landlocked salmon (*Salmo salar L.*) parr. Comp. Biochem. Physiol. 134, 349–366.

Rowley, A.F., Knight, J., Lloyd-Evans, P., Holland, J.W., Vickers, P.J., 1995. Eicosanoids and their role in immune modulation in fish-a brief overview. Fish Shellfish Immunol. 5, 549–567.

Ruyter, B., Moya-Falcón, C., Rosenlund, G., Vegusdal, A., 2006. Fat content and morphology of liver and intestine of Atlantic salmon (*Salmo salar*): Effects of temperature and dietary soybean oil. Aquaculture 252, 441–452.

Sheldon, J.W.M., Blazer, V.S., 1991. Influence of dietary lipid and temperature on bactericidal activity of channel catfish macrophages. J. Aquat. Anim. Health 3, 87–93.

Singh, R.K., Tandon, R., Dastidar, S.G., 2013. A. Ray, A review on leukotrienes and their receptors with reference to asthma. J. Asthma 50, 922–931.

Sun, D., Funk, C.D., 1996. Disruption of 12/15-lipoxygenase expression in peritoneal macrophages. Enhanced utilization of the 5-lipoxygenase pathway and diminished oxidation of low density lipoprotein. J. Biol. Chem. 271, 24055–24062.

Torstensen, B.E., Espe, M., Stubhaug, I., Lie, Ø., 2011. Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 106, 633–647.

Waagbø, R., 1994. The impact of nutritional factors on the immune system in Atlantic salmon, *Salmo salar L.*: a review. Aquac. Res. 25, 175–197.

Yao, C.L., Kong, P., Wang, Z.Y., Ji, P.F., Cai, M.Y., Liu, X.D., Han, X.Z., 2008. Cloning and expression analysis of two alternative splicing toll-like receptor 9 isoforms A and B in large yellow croaker, Pseudosciaena crocea. Fish Shellfish Immunol. 25, 648–656.

Yao, C.L., Kong, P., Wang, Z.Y., Ji, P.F., Liu, X.D., Cai, M.Y., Han, X.Z., 2009. Molecular cloning and expression of MyD88 in large yellow croaker, Pseudosciaena crocea. Fish Shellfish Immunol. 26, 249–255.

Yoshimoto, T., Takahashi, Y., 2002. Arachidonate 12-lipoxygenases. Prostaglandins Other Lipid Mediat. 68, 245–262.

Yuan, Y., Li, S., Mai, K., Xu, W., Zhang, Y., Ai, Q., 2015. The effect of dietary arachidonic acid (ara) on growth performance, fatty acid composition and expression of ara metabolism-related genes in larval half-smooth tongue sole (*Cynoglossus semilaevis*). Br. J. Nutr. 113 (10), 1518–1530.

Zuo, R.T., Ai, Q.H., Mai, K.S., Xu, W., Wang, J., Xu, H.G., Liufu, Z.G., Zhang, Y.J., 2012. 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, 101–109.

Zuo, R.T., Mai, K.S., Xu, W., Dong, X.J., Ai, Q.H., 2015. Dietary ALA, But not LNA, Increase Growth, Reduce Inflammatory Processes, and Increase Anti-Oxidant Capacity in the Marine Finfish *Larimichthys crocea*. Lipids 50, 149–163.