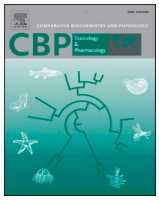
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Research article



Dietary docosahexaenoic acid reduces fat deposition and alleviates liver damage induced by D-galactosamine and lipopolysaccharides in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Liver health is important to maintain survival and growth of fish. Currently, the role of dietary docosahexaenoic acid (DHA) in improving fish liver health is largely unknown. This study investigated the role of DHA supplementation in fat deposition and liver damage caused by D-galactosamine (D-GalN) and lipopolysaccharides (LPS) in Nile tilapia (*Oreochromis niloticus*). Four diets were formulated as control diet (Con), Con supplemented with 1 % DHA, 2 % DHA and 4 % DHA diets, respectively. The diets were fed to 25 Nile tilapia (2.0 ± 0.1 g, average initial weight) in triplicates for four weeks. After the four weeks, 20 fish in each treatment were randomly selected and injected with a mixture of 500 mg D-GalN and 10 μL LPS per mL to induce acute liver injury. The results showed that the Nile tilapia fed on DHA diets decreased visceral somatic index, liver lipid content and serum and liver triglyceride concentrations than those fed on the Con diet. Moreover, after D-GalN/LPS injection, the fish fed on DHA diets decreased alanine aminotransferase and aspartate transaminase activities in the serum. The results of liver qPCR and transcriptomics assays together showed that the DHA diets feeding improved liver health by downregulating the expression of the genes related to toll-like receptor 4 (TLR4) signaling pathway, inflammation and apoptosis. This study indicates that DHA supplementation in Nile tilapia alleviates the liver damage caused by D-GalN/LPS through increasing lipid catabolism, decreasing lipogenesis, TLR4 signaling pathway, inflammation, and apoptosis. Our study provides novel knowledge on the role of DHA in improving liver health in cultured aquatic animals for sustainable aquaculture.

1. Introduction

Aquatic products are known as an important source of protein food for human being, which is rich in nutrition and popular among different people all over the world. The importance of aquatic products has promoted the development of the aquaculture industry. Currently, high-energy diets are widely used in aquaculture (Limbu et al., 2019; Li et al., 2020) in order to further improve the economic efficiency and shorten the production cycle. However, high-energy diets, especially the diets containing high level of protein and lipid, in fish increase toxic water quality parameters such as un ionized ammonia and nitrite (Heinen et al., 1996), leading to stress on the cultured animals. Moreover, long-term energy imbalance (Du et al., 2006), environmental pollution (Sun et al., 2020) and antibiotics abuse (Limbu et al., 2018) further aggravate the stress on the fish, which induce a series of metabolic syndromes diseases, especially fat deposition and impaired liver function (Guo et al., 2020; Zhou et al., 2020). Liver function damage leads to a series of negative effects including death. Therefore, finding effective approaches to alleviate metabolic diseases in fish, improve liver health, and provide high-quality aquatic products for human consumption is important for sustainability of aquaculture industry. Currently, aquatic nutritionists are focusing on the roles of various feed additives on fish liver health.

Docosahexaenoic acid (DHA, 22:6n-3) is one of the n-3 highly

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unsaturated fatty acids (HUFAs). DHA has various critical physiological functions, such as immune regulation, anti-inflammatory, antioxidant and lipid metabolism regulation (Juman et al., 2013; Lorente-Cebrián et al., 2013). DHA shows more powerful anti-inflammatory and antioxidant effects compared to other types of HUFAs (Chen et al., 2012; Enguita et al., 2019). DHA indicates its anti-inflammatory functions through a variety of pathways under different conditions. These include by targeting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways thereby inhibiting the production of pro-inflammatory cytokines in the liver (Chen et al., 2012). In addition, DHA as a natural ligand of peroxisome proliferator activated receptor-γ (PPARγ) (Gani, 2008), has been reported to inhibit the expression of transforming growth factor beta (TGFβ) and reduce inflammatory response in the liver through PPARγ activation (He et al., 2019; Jeon et al., 2014). In addition to alleviating the inflammatory response, DHA also plays an important role in reducing fat deposition in the liver (Soni et al., 2015; Tsuzuki et al., 2006; Wei et al., 2021). Therefore, DHA plays significant roles as an anti-inflammatory, antioxidant and has functions in lipid metabolism in fish.

Currently, DHA is usually supplemented into diets to improve growth performance, regulate lipid metabolism and improve antioxidant capacity of cultured fish. A previous study showed that supplementing 4 % DHA in the diet increased survival rate of the California halibut (*Paralichthys californicus*) and reduced malpigmentation (Vizcaíno-Ochoa et al., 2010). Moreover, adding 7.9 % and 12.6 % DHA in the diet increased weight gain and reduced hepatosomatic index (HSI) in juvenile cobia (*Rachycentron canadum*) (Trushenski et al., 2012). Furthermore, milkfish fed on DHA-enriched live feed increased survival rate compared with fish fed an unenriched diet (Gapasin and Duray, 2001). Similarly, dietary supplementation with 1 % DHA increased weight gain and reduced liver fat deposition and inflammation response in a freshwater herbivorous fish, grass carp (*Ctenopharyngodon idella*) (Huang et al., 2022). However, the metabolic regulation of DHA in omnivorous fish has been poorly studied. Moreover, the possible protective effect of DHA on acute liver injury has also not been well studied in fish.

Nile tilapia (*Oreochromis niloticus*) is warm-water omnivorous fish, which is widely farmed in the world (FAO, 2022; Li et al., 2022; Zhou et al., 2022). Traditionally, Nile tilapia have been considered to have a higher requirement for n-6 polyunsaturated fatty acid (n-6 PUFAs) in the diet such as linoleic acid (18:2 n-6, LOA) compared to n-3 PUFAs for maximal growth (Chen et al., 2013; Fernandes et al., 2019). However, some studies also pointed out that at low water temperature, the diet enriched in n-3 PUFAs (such as DHA) improved growth and feed efficiency in Nile tilapia (Corrêa et al., 2017; Nobrega et al., 2019). However, the high cost of DHA limits its long-term usage as a regular dietary component in diets due to relatively low Nile tilapia market price. Nevertheless, if DHA could efficiently improve health and prevent acute liver damage during short time supplementation in Nile tilapia, its use as a dietary functional feed additive might be economically justified.

Therefore, the present study evaluated the role of dietary DHA supplementation as a short-term used feed additive on lipid metabolism and liver damage in Nile tilapia. Nile tilapia were fed a control diet (Con), Con +1 % DHA supplemented diet (1 % DHA), Con +2 % DHA supplemented diet (2 % DHA) and Con +4 % DHA supplemented diet (4 % DHA) for four weeks. After the four weeks, 20 fish in each treatment were randomly selected and injected with a mixture of D-galactosamine (500 mg D-GalN) and lipopolysaccharides (10 μL LPS per mL of saline) to induce acute liver injury. The Nile tilapia fed on the Con diet were injected with an equal volume of saline as a negative control. The acute liver injury enduring test lasted for seven days. After the two experiments (feeding trial and acute liver injury), growth performance, biochemical indicators and expression of genes related to lipid metabolism and liver health were analyzed. The results obtained improve our understanding on the function of DHA in liver metabolism and health maintenance in cultured fish for sustainable aquaculture production.

2. Materials and methods

2.1. Animal ethics statement

All experiments were conducted under the Guidance of the Care and Use of Laboratory Animals in China. This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (Approval ID: f20201002).

2.2. Experimental fish, diets and study design

Five hundred Nile tilapia used in this experiment were purchased from Nanhai Tongwei Aquatic Technology Co., Ltd. (Foshan, China). Before the formal experiment, all fish were acclimated into two 500 L tanks under suitable water quality conditions for two weeks. During this time, the fish were fed on a commercial diet containing 350 g kg^{-1} protein and 5 g kg^{-1} lipid (Tongwei, Co. Ltd., Chengdu, China).

Four experimental feeds, named as control diet (Con, approximately protein 350 g kg^{-1}, lipid 6 g kg^{-1}), Con +1 % DHA supplemented diet (1 % DHA), 2 % DHA supplemented diet (2 % DHA), and 4 % DHA supplemented diet (4 % DHA) were formulated as shown in Table 1. Highly purified fish oil (85 % DHA, Shouhe Biological Technology Co. LTD, Xi'an, China) are used to replenish the DHA in the diets. The feeds were then dried by using a freeze dryer (Scientz-30ND, Ningbo, China). After drying, the feeds were stored at −30 °C until needed for feeding Nile tilapia during the study.

After the acclimation, 300 visually healthy Nile tilapia (average initial weight, 2.0 ± 0.1 g) were randomly selected and stocked into 200-L tank (three replicates per treatment, each 25 fish). The fish were fed the four experimental diets at a feeding rate of 4 % average body weight twice a day at 09:00 and 17:00 for four weeks in an indoor recirculating experimental system. The fish in each tank were bulk weighed every 10 days, and 8 days for the last sampling. After each sampling, the feeding amount was adjusted accordingly. During the whole study period, water temperature was maintained at 27.50 ± 1.00 °C, pH varied from 7.5 to 7.9, dissolved oxygen was not <6.2 mg/L, and total ammonia was <0.2 mg/L.

Table 1
Formulations of the experimental diets.

Component (g kg^{-1})	Con	1%DHA	2%DHA	4%DHA
Casein	344.00	344.00	344.00	344.00
Gelatin	86.00	86.00	86.00	86.00
Soybean oil	70.00	58.22	46.47	22.94
Fish oil (85 % DHA)	0	11.78	23.53	47.06
Corn starch	300.00	300.00	300.00	300.00
Vitamin premix[a]	15.00	15.00	15.00	15.00
Mineral premix[b]	45.00	45.00	45.00	45.00
$Ca(H_2PO_4)_2$	15.00	15.00	15.00	15.00
Carboxymethylcellulose	30.00	30.00	30.00	30.00
Dimethyl-β-propiothetin	2.00	2.00	2.00	2.00
Cellulose	87.75	87.75	87.75	87.75
Choline chloride	5.00	5.00	5.00	5.00
Butylated hydroxytoluene	0.25	0.25	0.25	0.25
Total	1000	1000	1000	1000
Proximate composition (% dry matter)				
Dry matter	86.90	92.40	91.80	93.70
Crude protein (%)	42.00	42.90	42.60	42.10
Crude lipid (%)	7.60	7.30	7.90	7.60

[a] Mineral premix, (g kg^{-1}): 314.0 g CaCO3; 469.3 KH2PO4; 147.4 g MgSO4·7H2O; 49.8 g NaCl; 10.9 g Fe (II) gluconate; 3.12 g MnSO4·H2O; 4.67 g ZnSO4·7H2O; 0.62 g CuSO4·5H2O; 0.16 g KJ; 0.08 g CoCl2·6H2O; 0.06 g NH4 molybdate; 0.02 g NaSeO3.

[b] Vitamin premix, (mg or IU/kg): 500,000 I.U. (international units) Vitamin A, 50,000 I.U. Vitamin D3, 2500 mg Vitamin E, 1000 mg Vitamin K3, 5000 mg Vitamin B1, 5000 mg Vitamin B2, 5000 mg Vitamin B6, 5000 μg Vitamin B12, 25,000 mg Inositol, 10,000 mg Pantothenic acid, 100,000 mg Choline, 25,000 mg Niacin, 1000 mg Folic acid, 250 mg Biotin, 10,000 mg Vitamin C.

At the end of the feeding trial, 20 fish from each treatment group were randomly selected for acute liver injury trial. Each individual fish was injected with a mixture of 500 mg D-GalN and 10 μL LPS per mL at a rate of 1 μg/g body weight once every two days for a total of three injections (day one, four and seven). The Nile tilapia fed on the Con diet were injected with D-GalN/LPS termed as CD treatment (Positive control). The Nile tilapia fed on the Con diet were injected with an equal volume of saline as a negative control (CC treatment). The acute liver injury experiment lasted for seven days.

2.3. Estimation of growth performance, blood collection and body indexes after the feeding trial

After the four weeks, all fish in each tank were fasted overnight. A sample of 12 fish in each treatment (four fish per tank) were randomly collected and then weighed to obtain final body weight to calculate weight gain (WG) by using the formula:

$$\text{WG}\ (\%) = 100 \times (\text{Final mean body weight} - \text{Initial mean body weight})/\text{Initial mean body weight}$$

Then, the 12 fish were euthanized by using MS-222 (tricaine methanesulfonate, Sigma, USA). Blood was quickly collected from caudal vein of individual fish by using 1 mL syringes (Klmedical, China), and then centrifuged at 1500 rpm, 4 °C for 10 min to obtain the serum. The serum obtained was stored at −80 °C for biochemical analysis as indicated in the next section. After blood collection, individual fish were dissected to obtain viscera, liver, and mesenteric fat. The viscera, liver, and mesenteric fat obtained from individual fish were weighed to determine the viscera somatic index (VSI), HSI, and mesenteric fat index (MFI) by using the following formulae:

$$\text{VSI}\ (\%) = 100 \times (\text{Viscera somatic weight}/\text{Individual body weight})$$

$$\text{HSI}\ (\%) = 100 \times (\text{Liver weight}/\text{Individual body weight})$$

$$\text{MFI}\ (\%) = 100 \times (\text{Mesenteric fat weight}/\text{Individual body weight})$$

After the HSI calculation, the liver sample from each individual fish was divided into different tubes and instantly frozen into liquid nitrogen, and stored at −80 °C until used for further *biochemical* analysis. Some of the remaining fish in each treatment after the 12 fish sampling were used for acute liver injury experiment as described in the next section.

2.4. Fatty acid composition analysis of the diets and fish liver after the feeding trial

The fatty acids composition of the experimental diets and Nile tilapia liver (20 mg) were analyzed by using Gas Chromatographic Mass Spectrometer (GCMS-QP2010, Shimadzu, China). Briefly, 2 mg fat for fatty acid methyl esters was obtained by using the chloroform/methanol (2/1, v/v) method (Folch et al., 1957). Total fat was prepared for saponification by adding 0.5 mL of methanolic KOH (2 mol/L) at 65 °C for 30 min. Subsequently, 14 % boron trifluoride-methanol was added for fatty acids methyl esters at 75 °C for 30 min, and then 1 mL hexane and 1 mL Milli-Q water were added. The mixture was centrifuged at 2000 rpm for 5 min, then the upper layers were collected for fatty acids analysis by using GCMS-QP2010 (Shimadzu, China). Fatty acids were identified by GCMS real-time analysis software (Shimadzu, China) and the peak area was used to represent the proportion of a specific fatty acid in the total fatty acids. The fatty acids composition of the experimental diets is shown in Table 2.

2.5. Lipid content and biochemical analyses

For the feeding trial, part of the liver sample from the 12 fish was used for determination of lipid content. The lipid content in the liver and experimental diets was determined by the chloroform/methanol (2/1, v/v) method (Folch et al., 1957). Part of the liver and the serum sampled above from the 12 fish were used for biochemical analysis for understanding the liver health and antioxidant capacity of fish. The triglyceride (TG) content, alanine aminotransferase (ALT), aspartate transaminase (AST), catalase (CAT) and total superoxide dismutase (SOD) activities in the serum were determined by using specific commercial assay kits (Nanjing Jiancheng Biotech Co., Nanjing, China) based on the manufacturer's instructions. Similarly, TG, glutathione (GSH) and malondialdehyde (MDA) contents, total antioxidant capacity (T-AOC), SOD, and CAT activities in the liver were also determined by using specific commercial assay kits (Nanjing Jiancheng Biotech Co., Nanjing, China).

For the acute liver injury trial, after the last injection, all fish were fasted for 24 h. Then, 12 fish were randomly selected in each treatment for serum and liver samples collection as described above. The ALT, AST, CAT and SOD activities in the serum were determined as described above. Moreover, T-AOC and CAT activities and GSH and MDA contents in the liver were also determined as described above.

Table 2
Fatty acid composition of the experimental diets.

Fatty acid (%)	CON	1%DHA	2%DHA	4%DHA
C12:0	0.05 ± 0.05^{b}	0.05 ± 0.05^{b}	0.31 ± 0.02^{a}	0.05 ± 0.06^{b}
C14:0	1.15 ± 0.48^{a}	0.97 ± 0.04^{b}	1.05 ± 0.03^{ab}	1.02 ± 0.06^{ab}
C15:0	0.14 ± 0.07	0.05 ± 0.05	0.13 ± 0.00	0.14 ± 0.01
C16:0	12.75 ± 0.13^{a}	10.73 ± 0.25^{b}	9.28 ± 0.26^{c}	6.67 ± 0.21^{d}
C16:1	0.35 ± 0.02	0.28 ± 0.02	0.28 ± 0.02	0.28 ± 0.02
C17:0	0.19 ± 0.01^{a}	0.12 ± 0.01^{bc}	0.14 ± 0.00^{b}	0.11 ± 0.01^{c}
C18:0	5.37 ± 0.37^{a}	4.74 ± 0.17^{ab}	4.31 ± 0.03^{b}	2.76 ± 0.08^{c}
C18:1n9t	22.14 ± 0.18^{a}	18.91 ± 0.15^{b}	15.98 ± 0.14^{c}	10.00 ± 0.14^{d}
C18:1n9c	1.30 ± 0.55	1.31 ± 0.07	1.11 ± 0.07	0.46 ± 0.21
C18:2n6t	n.d.	n.d.	0.04 ± 0.04	n.d.
C18:2n6c	46.05 ± 1.04^{a}	39.12 ± 0.42^{b}	32.09 ± 0.91^{c}	19.53 ± 0.46^{d}
C20:0	0.30 ± 0.04^{a}	0.28 ± 0.02^{a}	0.32 ± 0.07^{a}	0.13 ± 0.01^{b}
C18:3n3	8.57 ± 0.14^{a}	5.01 ± 2.51^{ab}	4.04 ± 2.02^{ab}	2.31 ± 0.03^{b}
C20:3n3	0.15 ± 0.15	2.55 ± 2.55	1.92 ± 1.92	1.06 ± 1.06
C20:4n6	n.d.	0.26 ± 0.26	0.15 ± 0.43	0.39 ± 0.24
C20:5n3	n.d.^{a}	0.64 ± 0.21^{ab}	1.20 ± 0.43^{b}	3.44 ± 0.12^{c}
C22:6n3	1.18 ± 0.05^{d}	14.74 ± 0.56^{c}	27.45 ± 0.54^{b}	51.40 ± 0.71^{a}
C22:0	0.25 ± 0.04^{a}	0.24 ± 0.02^{a}	0.22 ± 0.01^{a}	0.07 ± 0.04^{b}
C24:0	0.03 ± 0.03	n.d.	n.d.	0.02 ± 0.02
SFA	20.24 ± 0.54^{a}	17.19 ± 0.27^{b}	15.77 ± 0.20^{c}	10.98 ± 0.13^{d}
MUFA	23.79 ± 0.64^{a}	20.50 ± 0.21^{b}	17.37 ± 0.07^{c}	10.74 ± 0.17^{d}
n-6 PUFA	46.05 ± 1.04^{a}	39.38 ± 0.67^{b}	32.27 ± 0.84^{c}	19.92 ± 0.44^{d}
n-3 PUFA	9.91 ± 0.09^{d}	22.94 ± 0.87^{c}	34.62 ± 1.08^{b}	58.21 ± 0.40^{a}
n-3/n-6 PUFA	0.22 ± 0.00^{d}	0.58 ± 0.03^{c}	1.08 ± 0.06^{b}	2.92 ± 0.08^{a}

In the same row, values that are not followed by the same letter are significantly different ($p<0.05$).

2.6. Histological analysis

After the feeding trial, three liver tissue samples from each treatment were fixed with paraformaldehyde solution and sent to Wuhan Servicebio Technology Co., Ltd. for oil-red and H & E staining after 24 h of fixation. Before staining, the fixed liver samples were embedded in paraffin. Sections with 5 μm thickness were stained with the hematoxylin-eosin and oil red O and then washed with 70 % alcohol. The stained samples were examined under a light microscope (Nikon Ds Ri2, Tokyo Metropolis, Japan). Histological analysis of the liver tissue after acute liver injury trial was consistent with the above.

2.7. Quantitative real-time PCR

After the feeding trial, we analyzed the effects of dietary DHA on mRNA expression of genes related to lipid metabolism. Total RNA from eight fish in each treatment was extracted from the frozen liver tissues (approximately 20 mg) by using Trizol reagent (Beyotime, Shanghai, China) according to the manufacture's protocol. The quality and quantity of total RNA were estimated by the 260/280 nm absorbance ratio by using a Nano Drop 2000 Spectrophotometer (Thermo, USA). The cDNAs were synthesized by using a PrimerScript™ RT reagent Kit (No. R323–01, Vazyme Biotech Co, Nanjing, China) following the manufacturer's instructions. The real-time quantitative PCR (qRT-PCR) was conducted by using CFX96 real-time RCR system (Bio-Rad, Richmond, CA) as described previously in our laboratory (Lu et al., 2019). The β-actin (*β-actin*) and elongation factor 1α (*ef1α*) were used as reference genes due to their stability. The primers used for genes related to lipid metabolism are shown in Table 3. The cDNA expression of genes was estimated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

After the acute liver injury trial, we analyzed the genes related to the toll-like receptor 4 (TRL4) signaling pathway, inflammation, apoptosis and antioxidant capacity by using the methods described above. The primers for the genes related to TRL4 signaling pathway, inflammation, apoptosis and antioxidant capacity are shown in Table 3.

2.8. Liver transcriptome sequencing analysis after acute liver injury

Total RNA was extracted from the liver tissue (20 mg) by using Trizol Reagent (Invitrogen) according the manufacturer's instructions and genomic DNA was removed using DNase I (TaKara). The RNA quality was determined by using 2100 Bioanalyser (Agilent) and quantified by using the ND-2000 (NanoDrop Technologies). Only high-quality RNA samples (OD260/280 = 1.8– 2.2, OD260/230 ≥ 2.0, RIN ≥ 6.5, 28S:18S ≥ 1.0, >1 μg) were used to construct sequencing library.

RNA-seq transcriptome library was prepared following TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA) by using 1 μg of total RNA. Briefly, mRNA was isolated according to polyA selection method by oligo(dT) beads and then fragmented by fragmentation buffer. The double-stranded cDNA was synthesized by using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). Then the synthesized cDNA was subjected to end-repair, phosphorylation and 'A' base addition according to Illumina's library construction protocol. Libraries were selected for cDNA target fragments based on size of 300 bp on 2 % Low Range Ultra Agarose followed by PCR amplified using Phusion DNA polymerase (NEB) for 15 PCR cycles. The paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq xten/NovaSeq 6000 sequencer (2 × 150 bp read length) after quantification by using Turner BioSystems (TBS380) Mini-Fluorometer.

All sequencing data were uploaded to bioinformation cloud platform of Majorbio for further analysis (www.majorbio.com). The DEGSeq2 R package was used to analyze the differential expression genes (DEGs) among CC, CD and DHA treatments. A *P*-value < 0.05 and |log2 (fold change)| > 2 gene were considered as differentially expressed.

Table 3
Primers used for RT-qPCR expression analysis.

Gene	Primer (5′-3′)	GenBank No.
ef1α	F: ATCAAGAAGATCGGCTACAACCCT R: ATCCCTTGAACCAGCTCATCTTGT	KJ123689.1
β-actin	F: AGCCTTCCTTCCTTGGTATGGAAT R: TGTTGGCGTACAGGTCCTTACG	KJ126772.1
tlr5	F: GGCAAAGATCCCAGCCTGTA R: ACATTCTGTCCTCAATCACCCT	XM_025898784.1
myd88	F: TTTACGCTTCCTCACCATTTGT R: CCGCCTGCTCCACAGTTAT	XM_030756572.1
irak4	F: CTGTCATCAGACAGGTTTACGC R: GCAAGCTCAGAATTCCCACG	XM_003443911.5
irak1α	F: AAACGGCTGATTTCAGGGGG R: ACGTCCTCAAGAGATGCACA	XM_003457627.5
traf6	F: TGATCCACCGCTGGAGAGTA R: CTGTCCCGTATCACGGATGG	XM_003450798.5
nf-κb	F: CGACCACTACCTACACGCTC R: GATGTCGTTTGAGGCATCGC	XM_019363515.2
il1β	F: GAGCACAGAATTCCAGGATGAAAG R: TGAACTGAGGTGGTCCAGCTGT	XM_019365841.1
il8	F: CTGTGAAGGCATGGGTGTGGAG R: TCGCAGTGGGAGTTGGGAAGAA	NM_001279704.1
il6	F: ACAGAGGAGGCGGAGATG R: GCAGTGCTTCGGGATAGAG	XM_019350387.2
il12	F: AGGTCAGCCAATCTGTGCCACT R: CCGTGATGTTCTGGAGCAGTGTTC	XM_003437924.4
sod1	F: GCCCACACTTCAATCCCTACAA R: GGCTCTCTTCATTTCCTCCTTT	XM_003446807.4
gsh-px	F: ACCTTCATTCTCGCTACTCC R: GCAGTTCTCCTGATGTCCAA	XM_039611806.1
gst	F: TAATGGGAGAGGGAAGATGG R: CTCTGCGATGTAATTCAGG	NM_001279635.1
caspase8	F: AACTGGAATCTTCTGAGGTGGCA R: TTGAGAAGAGATCTTTGGCCTGC	XM_005475926.3
caspase9	F: ATACTTGAGGAAAACGCTGCCACT R: GAACCAGGCATTTGTTTGTAGAGC	XM_003455320.4
caspase3	F: GGAGTGGACGATACAGACGCAAA R: TGAAGCTGTGTGACTGGGGCTT	NM_001282894.1

2.9. Statistical analyses

All data were tested for normality by using Shapiro-Wilk test and homogeneity of variances by using Levene's test, except those for histology and transcriptome analyses. The significant differences on measured parameters among the experimental diets after the feeding trial were evaluated by using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for specific comparisons. The results with $P \leq 0.05$ were considered statistically significant. The significant differences for the measured parameters after acute liver injury were analyzed by using independent-samples *t*-test. Significance differences after acute liver injury were determined between CC and CD treatments; and CD and DHA treatments. Results with $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ were considered statistically significant. During reporting, the results are expressed as mean ± standard error of the mean (SEM). All data were analyzed by using SPSS Statistics 20.0 software (IBM, Armonk, NY, USA).

3. Results

3.1. Effects of dietary DHA on growth performance and organ indexes

Results on the growth performance of the Nile tilapia fed on the four experimental diets are presented in Fig. 1. Feeding the Nile tilapia with the experimental diets did not affect significantly the weight increase (Fig. 1A), WG (Fig. 1B), HSI (Fig. 1C) and MFI (Fig. 1E) ($P > 0.05$). However, the fish fed on dietary 4 % DHA decreased significantly the VSI than those fed on the Con diet ($P < 0.05$; Fig. 1D). The fish fed on Con diet and those fed on 1 % and 2 % DHA, on one hand, and those fed on 4 % DHA diet and 1 % and 2 % DHA diets, on the other hand, had statistically comparable VSI values ($P > 0.05$).

3.2. Effects of dietary DHA on fatty acid composition in the liver

The fish fed on the DHA diets increased the DHA proportion in the liver in a dose-dependent manner compared to those fed on the Con diet ($P < 0.05$; Table 4). However, the fish fed on 2 % DHA diet has no significant difference in DHA levels to those fed on 1 % and 4 % DHA diets ($P > 0.05$). Moreover, feeding the Nile tilapia with the DHA diets did not affect the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) proportions in the liver ($P > 0.05$). Interestingly, the fish fed on the dietary DHA levels increased the proportion of n-3 PUFA, but decreased the proportion of n-6 PUFA than those fed on the Con diet

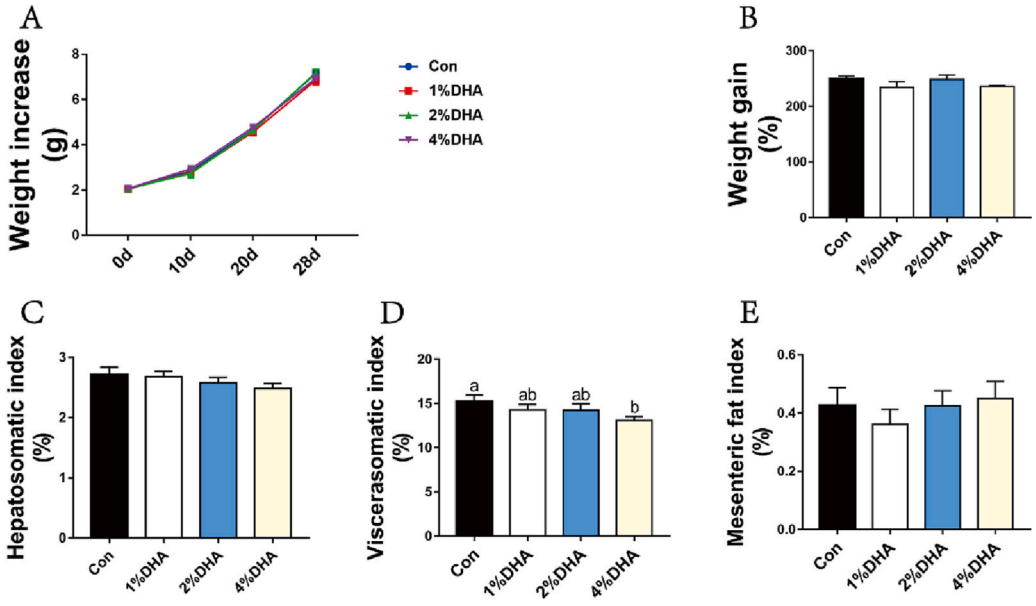


Fig. 1. Effects of dietary DHA on growth performance and organ indexes. (A) Weight increase; (B) Weight gain (WG); (C) Hepatosomatic index (HSI); (D) Viscerosomatic index (VSI); and (E) Mesenteric fat index (MFI). Results are presented as mean ± SEM (n = 12). Mean values with different superscript letters indicate statistical difference ($P < 0.05$).

Table 4
Fatty acid composition in the liver of Nile tilapia.

Fatty acid (%[1])	CON	1%DHA	2%DHA	4%DHA
C14:0	4.09 ± 0.10^{a}	3.21 ± 0.25^{b}	3.62 ± 0.27^{ab}	3.11 ± 0.27^{b}
C14:1	0.03 ± 0.03	0.12 ± 0.04	0.10 ± 0.06	0.18 ± 0.08
C15:0	n.d.	n.d.	n.d.	n.d.
C16:0	19.66 ± 0.91	19.97 ± 1.73	19.94 ± 1.48	19.36 ± 0.75
C16:1	6.21 ± 0.48	7.50 ± 1.42	7.86 ± 1.24	6.87 ± 1.51
C17:0	0.37 ± 0.08	0.46 ± 0.05	0.39 ± 0.08	0.30 ± 0.06
C18:0	10.39 ± 0.27	9.94 ± 0.64	10.39 ± 0.58	10.40 ± 0.53
C18:1n9t	32.98 ± 0.81	29.04 ± 1.09	29.22 ± 1.92	27.17 ± 3.10
C18:1n9c	0.45 ± 0.17^{a}	n.d.	n.d.	0.29 ± 0.17^{ab}
C18:2n6t	1.21 ± 0.16^{a}	0.98 ± 0.38^{a}	0.09 ± 0.05^{b}	n.d.
C18:2n6c	12.45 ± 0.66^{a}	8.61 ± 2.79^{ab}	8.24 ± 1.17^{ab}	6.17 ± 0.36^{b}
C20:0	0.08 ± 0.08	0.16 ± 0.09	0.07 ± 0.07	0.21 ± 0.04
C18:3n6	1.29 ± 0.08^{a}	1.05 ± 0.04^{ab}	0.94 ± 0.12^{b}	0.53 ± 0.05^{c}
C20:1	1.84 ± 0.11	1.63 ± 0.23	1.44 ± 0.19	1.61 ± 0.24
C18:3n3	0.77 ± 0.06	0.77 ± 0.10	0.60 ± 0.16	0.46 ± 0.03
C20:3n6	1.19 ± 0.07	1.09 ± 0.25	1.13 ± 0.26	0.81 ± 0.15
C20:3n3	0.07 ± 0.07	0.12 ± 0.07	0.11 ± 0.07	0.13 ± 0.08
C20:4n6	5.31 ± 1.08	4.85 ± 0.93	4.04 ± 0.89	3.47 ± 0.67
C20:5n3	n.d.	n.d.	0.05 ± 0.05	0.21 ± 0.13
C22:6n3	1.63 ± 0.32^{c}	7.90 ± 0.17^{bc}	11.78 ± 1.80^{ab}	18.75 ± 4.12^{a}
SFA	34.58 ± 0.58	33.74 ± 1.19	34.40 ± 1.23	33.37 ± 0.50
MUFA	41.51 ± 1.26	38.28 ± 2.30	38.63 ± 2.93	36.11 ± 4.67
n-6 PUFA	21.45 ± 1.51^{a}	16.59 ± 3.32^{ab}	14.44 ± 2.22^{b}	10.97 ± 0.63^{b}
n-3 PUFA	1.70 ± 0.31^{c}	8.02 ± 1.24^{bc}	11.95 ± 1.84^{ab}	19.09 ± 4.32^{a}
n-3/n-6 PUFA	0.08 ± 0.02^{c}	0.54 ± 0.13^{bc}	0.83 ± 0.09^{b}	1.69 ± 0.29^{a}

Values in the same row with different superscript letters are statistically different ($P < 0.05$), while those without any superscript letter were not significant different ($P > 0.05$). Results are represented as mean ± SEM (n = 3).

(Table 4). Accordingly, the fish fed on 2 % and 4 % DHA diets had significantly higher n-3 PUFA, but significantly lower n-6 PUFA than those fed on the Con diet ($P < 0.05$). However, the fish fed on Con diet and those fed on 1 % DHA diet, on one hand, and those fed on 2 % and 4 % DHA diets and 1 % DHA diet, on the other hand, had no significant differences on n-3 PUFA and n-6 PUFA levels ($P > 0.05$). The n-3/n-6 PUFA ratio increased as the dietary DHA inclusion levels increased. The fish fed on dietary 4 % DHA had significantly higher n-3/n-6 PUFA ratio than those fed on the remaining three diets ($P < 0.05$). Similarly, the fish fed on 2 % DHA had significantly higher n-3/n-6 PUFA ratio than those fed on the Con diet ($P < 0.05$). However, the fish fed on Con diet and those fed on 1 % DHA diet, on one hand, and those fed on 1 % and 2 % DHA diets, on the other hand, had no significant differences on n-3/n-6 PUFA ratio ($P > 0.05$).

3.3. Effects of dietary DHA on liver health and antioxidant responses

The fish fed on dietary 2 % DHA reduced significantly ALT activity in serum than those fed on the Con diet ($P < 0.05$; Fig. 2A). However, the fish fed on Con diet and those fed on 1 % and 4 % DHA diets, on one hand, and those fed on 1 % and 2 % DHA diets and 4 % DHA diets, on the other hand, had no significant differences on ALT activity in the serum ($P > 0.05$). The Nile tilapia fed on the DHA diets decreased significantly AST activity in the serum than those fed on Con diet ($P < 0.05$; Fig. 2B). The fish fed on the DHA levels did not have significant differences in AST activity in the serum among them ($P > 0.05$). Moreover, feeding the Nile tilapia with the DHA diets did not affect significantly the SOD activity in serum (Fig. 2C), and T-AOC (Fig. 2D) and SOD (Fig. 2E) activities and MDA content (Fig. 2F) in the liver.

3.4. Effects of dietary DHA on lipid deposition

Feeding the Nile tilapia with dietary DHA levels decreased significantly the total lipid content in the liver (Fig. 3A) and TG content in the serum (Fig. 3C) compared to those fed on the Con diet ($P < 0.05$). However, the fish fed on the DHA diets did not have significant differences in total lipid and TG contents among them ($P > 0.05$). The Nile tilapia fed on 2 % and 4 % DHA diets decreased significantly the TG content in the liver (Fig. 3B) compared to those fed on the Con diet ($P < 0.05$). However, the fish fed on Con diet and those fed on 1 % DHA diet, on one hand, and those fed on 1 % DHA diet and 2 % and 4 % DHA diets, on the other hand, had no significant differences in TG content in the liver ($P > 0.05$). In addition, dietary DHA supplementation obviously reduced the number and size of lipid droplets in Nile tilapia liver compared to those fed the Con diet (Fig. 3D).

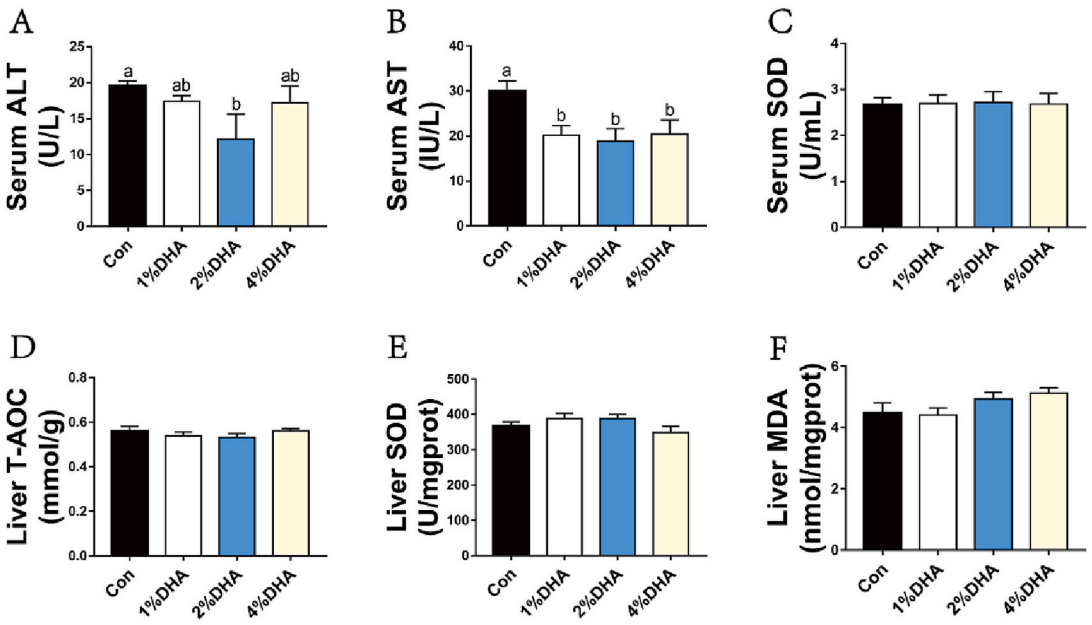


Fig. 2. Effects of dietary DHA on liver health and antioxidant response. (A) Serum alanine aminotransferase (ALT) activity; (B) Serum aspartate transaminase (AST) activity; (C) Serum total superoxide dismutase (SOD) activity; (D) Liver total antioxidant capacity (T-AOC) activity; (E) Liver SOD activity; and (F) Liver malondialdehyde (MDA) content. Results are presented as mean ± SEM (n = 4–6). Mean values with different superscript letters indicate statistical difference ($P < 0.05$).

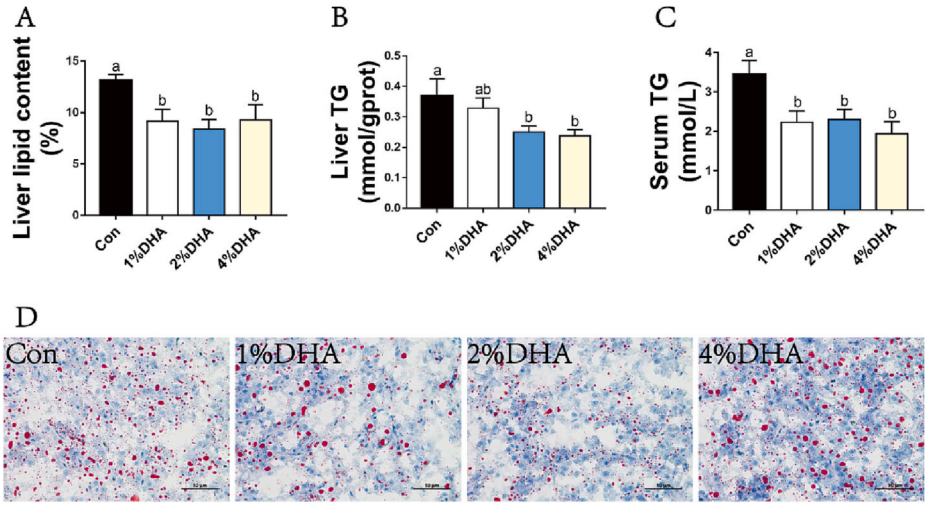


Fig. 3. Effects of dietary DHA on lipid metabolism. (A) Liver lipid content; (B) Liver triglyceride (TG) content; (C) Serum TG content; (D) Oil red staining of liver sections for fish fed on Con, 1 % DHA, 2 % DHA and 4 % DHA diets, respectively. Results are presented as mean ± SEM (n = 4–6). Mean values with different letters indicate statistical difference ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Effects of dietary DHA on expression of genes related to lipid metabolism

Feeding the Nile tilapia on the DHA diets up-regulated significantly most mRNA expression of the genes related to lipid catabolism such as adipose triglyceride lipase (*ATGL*), carnitine palmitoyltransferase 1 a (*CPT1a*), and peroxisome proliferator-activated receptor alpha (*PPARα*) ($P < 0.05$; Fig. 4A). However, feeding the Nile tilapia on the DHA diets did not affect mRNA expression of hormone-sensitive lipase (*HSL*), peroxisomal acyl-coenzyme A oxidase (*ACO*) and acetyl-CoA carboxylase beta (*ACCβ*) ($P > 0.05$). Feeding the Nile tilapia with the DHA diets down-regulated significantly most mRNA expression of genes related to lipogenesis, such as fatty acid synthase (*FAS*), acetyl coenzyme A carboxylase alpha (*ACCα*), diacylglycerol O-acyltransferase 2 (*DGAT2*), and sterol-regulatory element binding proteins 1 (*SREBP1*), compared with those fed on the Con diet ($P < 0.05$; Fig. 4B).

3.6. Effects of dietary DHA on D-GalN/LPS-induced liver injury in Nile tilapia

The fish in the CD treatment increased significantly ALT (Fig. 5A) and AST (Fig. 5B) activities in the serum than all other treatments after D-GalN/LPS injection ($P < 0.05$). Interestingly, the ALT (Fig. 5A) and AST (Fig. 5B) activities were significantly decreased to a similar level as the CC treatment in the DHA treatments ($P < 0.05$), except the AST activity for the 4 % DHA diet ($P > 0.05$). The results of H & E staining showed that the Nile tilapia injected with D-GalN/LPS (CD treatment) increased significantly the number and size of the vacuoles in the liver compared to the CC treatment (Fig. 5C). Interestingly, the Nile tilapia liver morphology in the DHA treatments was comparatively similar to the CC treatment (Fig. 5C).

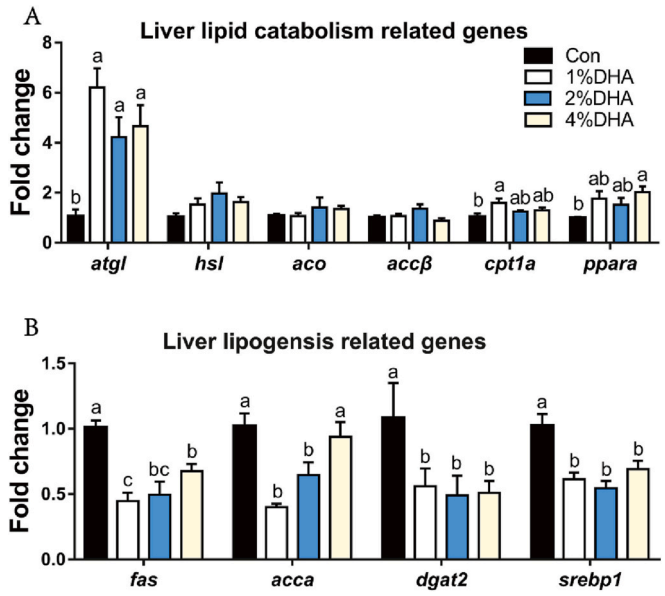


Fig. 4. Effects of dietary DHA on mRNA expression of genes related to lipid metabolism. (A) Lipolysis-related genes (ATGL = adipose triglyceride lipase, HSL = hormone-sensitive lipase, ACO = peroxisomal acyl-coenzyme A oxidase, ACCβ = acetyl-CoA carboxylase beta, CPT1α = carnitine palmitoyltransferase 1α, PPARα = peroxisome proliferator-activated receptor alpha); and (B) Lipogenesis-related genes (FAS = fatty acid synthase, ACCα = acetyl coenzyme A carboxylase alpha, DGAT2 = diacylglycerol O-acyltransferase 2, SREBP1 = sterol-regulatory element binding proteins 1). Results are presented as mean ± SEM (n = 3–6). Mean values for the same gene with different letters indicate statistical difference ($P < 0.05$).

3.7. Effects of dietary DHA on antioxidant responses in the fish with acute liver injury

The fish in the CD treatment decreased significantly SOD (Fig. 6A) and CAT (Fig. 6B) activities in the serum and T-AOC (Fig. 6C) and CAT (Fig. 6E) activities and GSH content (Fig. 6E) in the liver after D-GalN/LPS injection compared with the CC treatment ($P < 0.05$). Notably, the fish in the DHA treatments increased significantly SOD, CAT, T-AOC and CAT activities and GSH content similar to CC treatment. Meanwhile, the fish in the CD treatment increased significantly MDA content in the liver, a lipid peroxidation product, compared with the CC treatment ($P < 0.05$). Interestingly, DHA supplementation decreased significantly the MDA content in the Nile tilapia liver than the CD treatment ($P < 0.05$).

3.8. Effects of dietary DHA on expression of genes related to health in the fish with acute liver injury

The Nile tilapia in the CD treatment up-regulated significantly the expression of genes related to TLR4 pathway, such as toll-like receptor 5 (*TLR5*), myeloid differentiation primary response gene 88 (*MYD88*), interleukin 1 receptor associated kinase 4 (*IRAK4*) and TNF receptor associated factor 6 (*TRAF6*) in the liver than the CC treatment after D-GalN/LPS injection ($P < 0.05$; Fig. 7A). Interestingly, the expression of these genes was all down-regulated to a similar level as in the CC treatment after DHA supplementation ($P < 0.05$). Meanwhile, the Nile tilapia in the CD treatment up-regulated significantly inflammation-related genes such as *NF-κB*, interleukin 1 beta (*IL1β*), *IL8*, *IL6* and *IL12* in the liver compared with the CC treatment ($P < 0.05$; Fig. 7B). Astonishingly, all these genes were down-regulated significantly after DHA supplementation compared to CD treatment ($P < 0.05$). In addition, the Nile tilapia in the CD treatment also increased apoptosis initiator genes such as cysteine-aspartic acid protease 8 (*caspase8*) and *caspase9* and the effector gene *caspase3* compared to the CC treatment ($P < 0.05$; Fig. 7C). Interestingly, all these genes were down-regulated significantly after DHA supplementation compared to CD treatment ($P < 0.05$). Noticeable, there was no significant difference in liver antioxidant related genes (superoxide dismutase 1 solube, *SOD1*; glutathione peroxidase, *GSH-PX*; and glutathione s-transferases, *GST*) among the CC, CD and DHA treatments ($P > 0.05$; Fig. 7D).

3.9. Transcriptome analysis of liver after acute liver injury

The results of liver transcriptome analysis are presented in Fig. 8. The principal component analysis (PCA) showed clear separation among the treatments (Fig. 8A). The results of DEGs volcano-plots indicated that 173 genes were down-regulated significantly and 248 genes were up-regulated significantly in the liver of Nile tilapia in the CD treatment compared with the CC treatment (Fig. 8B). Moreover, 190 genes were down-regulated and 123 genes were up-regulated in the liver of Nile tilapia in the DHA treatment compared to the CD treatment (Fig. 8C). The results of clustering analysis showed that the DEGs levels in the liver of the CC treatment were consistent with DHA treatment, while the DEGs levels in the liver of Nile tilapia in the CD treatment were opposite to those in the CC and DHA treatments (Fig. 8D). Kyoto encyclopedia of genes and genomes (KEGG) functional annotation analysis of DEGs showed that a total of 26 signaling pathways were annotated including lipid metabolism, apoptosis, immunity and infectious disease (Fig. 8E). The heatmap revealed that the Uridine 5′-diphospho-glucuronosyl-transferase (UDP-glucuronosyltransferase, UGT), ceramide synthase 2 (CerS2a) and diacylglycerol kinase epsilon (DGKE) were the main lipid metabolism pathways changed (Fig. 8F). Moreover, Diablo was the main apoptosis pathway, FOS like 1, AP-1 transcription factor subunit a (fosl1a) was the main immunity pathway and insulin receptor substrate 2 (Irs2), MYC proto-oncogene (Myc), Trypsin2 and Dimannoside (Pim2) were the main DEGs signaling pathways significantly changed for infectious disease (Fig. 8F).

4. Discussion

In the present study, feeding the Nile tilapia on the different levels of DHA diets did not alter weight gain, HSI and MFI, although decreased the VSI, similar to GIFT Nile tilapia fed on 11.42 % to 16.20 % DHA (Luo et al., 2012). Generally, the growth-promoting effects of the n-6 PUFAs were superior to those of the n-3 PUFAs for tilapia (Fernandes et al., 2019). Several researches reported that n-3 FAs supplementation has no effect the growth performance of tilapia (Dos Santos et al., 2011; Luo et al., 2012), which is consistent with our present study. Furthermore, the optimal dietary requirements of n-3 long-chain PUFAs (LCPUFA), such as DHA, for tilapia have not been determined (Jackson et al., 2020). Nevertheless, the dietary supplementation of n-3 LCPUFAs in the diets still has potential benefits for tilapia, such as promoting muscle protein deposition and maintaining the balance of fatty acids in the liver and muscle (Duarte et al., 2021; Chou et al., 2001). Interestingly, we found that the DHA diets improved liver health in Nile tilapia by the decreasing ALT and AST activities in the serum, consistent with the results in grass carp serum (Huang et al., 2022). In addition, dietary DHA supplementation reversed liver damage by conjugated linoleic acid in mice and decreased the ALT activity in the serum (Adkins et al., 2016). However, the DHA supplementation did not affect SOD activity in serum and T-AOC and SOD activities and MDA content in the liver. These results suggest that, under normal feeding, DHA alleviates liver damage through transaminase but not through antioxidant capacity.

As expected, the DHA supplementation reduced the fat deposition in the liver and the TG content in the liver and serum. Accordingly, the expression of genes related to lipid catabolism such as *ATGL*, *CPT1a* and *PPARα* were upregulated while lipogenesis genes such as *FAS*, *ACCα*, *DGAT2*, and *SREBP1* were all downregulated by DHA supplementation, consistent with the previous fish findings in grass carp (Huang et al., 2022) and black sea bream (Ahmad Daud et al., 2003; Om et al., 2001)

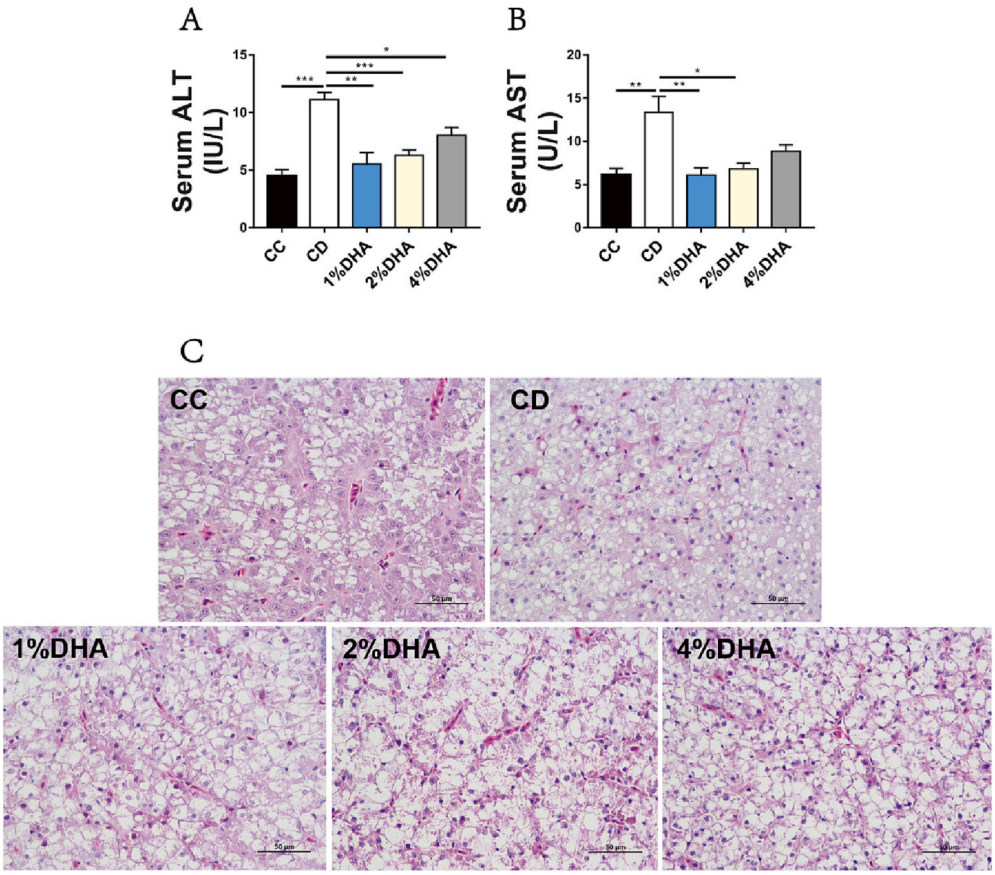


Fig. 5. Effect of dietary DHA on D-GalN/LPS-induced liver injury in Nile tilapia. (A) Serum alanine aminotransferase (ALT); and (B) Serum aspartate transaminase (AST) activities. Results are presented as mean ± SEM (n = 4–6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for significant differences between CC and CD treatments; and CD and DHA treatments. (C) H & E staining of liver sections for fish fed on Con, 1 % DHA, 2 % DHA and 4 % DHA diets.

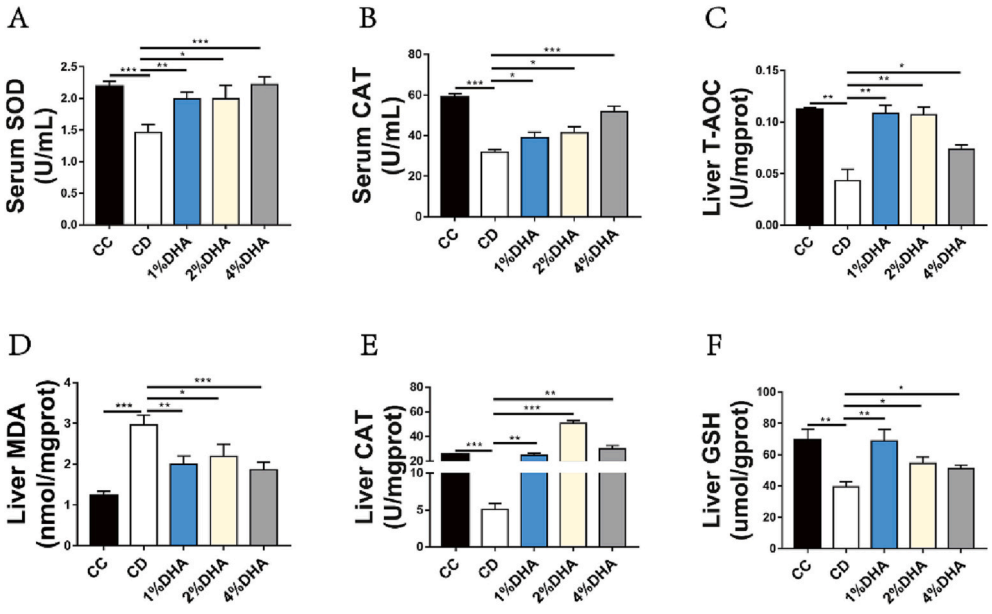


Fig. 6. Effect of dietary DHA on antioxidant response of liver injured Nile tilapia. (A) Serum superoxide dismutase (SOD) activity; (B) Serum catalase (CAT) activity; (C) Liver total antioxidant capacity (T-AOC) activity; (D) Liver malondialdehyde (MDA) content; (E) Liver CAT activity; and (F) Liver glutathione (GSH) content. Results are presented as mean ± SEM (n = 4–6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for significant differences between CC and CD treatments; and CD and DHA treatments.

and from some mammalian studies (Le et al., 2012; Soni et al., 2015; Tsuzuki et al., 2006). These results indicated dietary DHA supplementation reduced fat deposition in the liver by enhancing lipid catabolism and inhibiting lipid synthesis at the transcriptional level. Several studies have shown that lipid deposition in the liver can cause liver injury in fish (Dai et al., 2019; Liu et al., 2022; Wang et al., 2022). Therefore, dietary DHA supplementation improves liver health by reducing fat deposition.

DHA is one of the n-3 HUFAs and has various biological activities, such as immune regulation, anti-inflammatory, antioxidant and lipid metabolism regulation (Juman et al., 2013; Luo et al., 2012; Martínez-

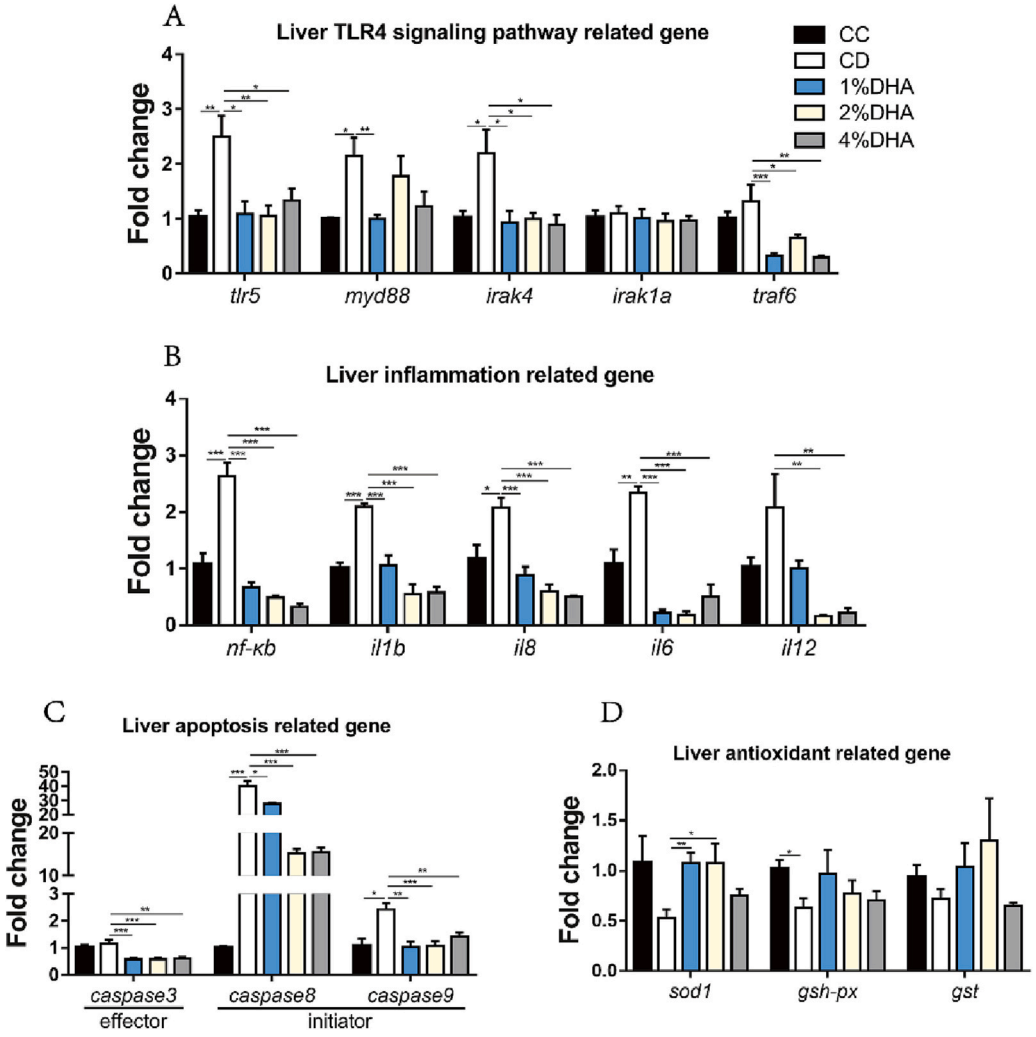


Fig. 7. Effect of dietary DHA on the expression of liver-related genes in liver injured Nile tilapia. (A) TLR4 signaling pathway-related genes (toll-like receptor 5, TLR5; myeloid differentiation primary response gene 88, MYD88; interleukin 1 receptor associated kinase 4, IRAK4; interleukin 1 receptor associated kinase 1 alpha, IRAK1α; and TNF receptor associated factor 6, TRAF6); (B) Inflammation-related genes (nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; interleukin 1 beta, IL1β; IL8; IL6; and IL12); (C) Apoptosis-related genes (cysteine-aspartic acid protease 8, caspase8; caspase9 and caspase3); and (D) Antioxidant-related genes (superoxide dismutase 1 solube, SOD1; glutathione peroxidase, GSH-PX; and glutathione s-transferases, GST). Results are presented as mean ± SEM (n = 4–6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for significant differences between CC and CD treatments; and CD and DHA treatments.

Fernández et al., 2015). Previous studies showed that DHA alleviated cholestatic liver injury (Chen et al., 2012), valproate-induced liver injury (El-Mowafy et al., 2016) and carbon tetrachloride-induced liver injury (He et al., 2017) in mice. Therefore, our present study assessed whether the application of DHA could alleviate the liver damage caused by D-GalN/LPS in Nile tilapia. The results indicated that D-GalN/LPS induced acute liver injury in Nile tilapia by increasing ALT and AST activities. Interestingly, dietary DHA supplementation decreased ALT and AST activities in the serum. Likewise, D-GalN/LPS injection reduced antioxidant-related enzymes in the serum and Nile tilapia liver and increased a lipid peroxidation product, MDA content in the liver. However, dietary DHA supplementation improved the serum and liver antioxidant capacity, reaching a basal level. Similarly, dietary supplementation with 1.5 % DHA increased T-AOC and CAT activities in common carp liver (Ji et al., 2009). In addition, DHA supplementation attenuated effectively the oxidative stress caused by liver injury in AML12 cells and mice (Chen et al., 2021). Therefore, our present results indicate that DHA supplementation alleviates D-GalN/LPS-induced liver damage in Nile tilapia by reducing transaminases and enhancing antioxidant activities.

D-GalN/LPS is a classical chemical that induces liver damage (Wu et al., 2019). LPS is the surface antigen substance of Gram-negative bacteria and *in vivo*, it activates the intracellular the mitogen-activated protein kinases (MAPKs)-NFκB signaling pathway by binding to the TLR4 protein and produces various inflammatory factors such as *IL-1*, *IL-6* and tumor necrosis factor alpha (TNF-α), leading to liver damage (Han et al., 2012). Our present study showed that injecting the Nile tilapia with D-GalN/LPS upregulated the mRNA expression of genes related to TLR4 pathway in liver such as *TLR5*, *MYD88*, *IRAK4* and *TRAF6*. Interestingly, dietary DHA supplementation reduced the expression of these genes. As expected, the expression of inflammation-related genes (such as *NF - κB*, *IL1β*, *IL8*, *IL6* and *IL12*) and apoptosis-related genes (such as *caspase3*, *caspase8* and *caspase9*) showed a similar trend in the liver of Nile tilapia in all three treatments. These results demonstrated that DHA reduced liver inflammation and alleviated liver damage by decreasing the expression of TLR4-NFκB signaling pathway. Many studies have reported on the regulation of TLR4 by fatty acids, and it is now generally accepted that SFAs activate TLR4, while HUFAs inhibit TLR4 (Hwang et al., 2016; Lee et al., 2003; Tang et al., 2018). A previous study showed that, DHA inhibits the dimerization and recruitment of TLR4 to lipid rafts, thereby blocks the association of TLR4 with myeloid differentiation factor 2 (MD-2) and downstream adaptor molecules, and NF-κB activation and target gene expression (Wong et al., 2009). Our present study showed that DHA also alleviates liver damage by inhibiting the TLR4 signaling pathway in Nile tilapia. However, the precise mechanism for the ability of DHA to alleviate liver damage requires further studies to explore.

The present study used the transcriptomic approach to analyze the effects of dietary DHA on Nile tilapia liver. Through KEGG functional annotation analysis, we focused on four signaling pathways, namely lipid metabolism, apoptosis, immune system and infectious disease that may be associated with DHA alleviation of D-GalN/LPS-induced liver

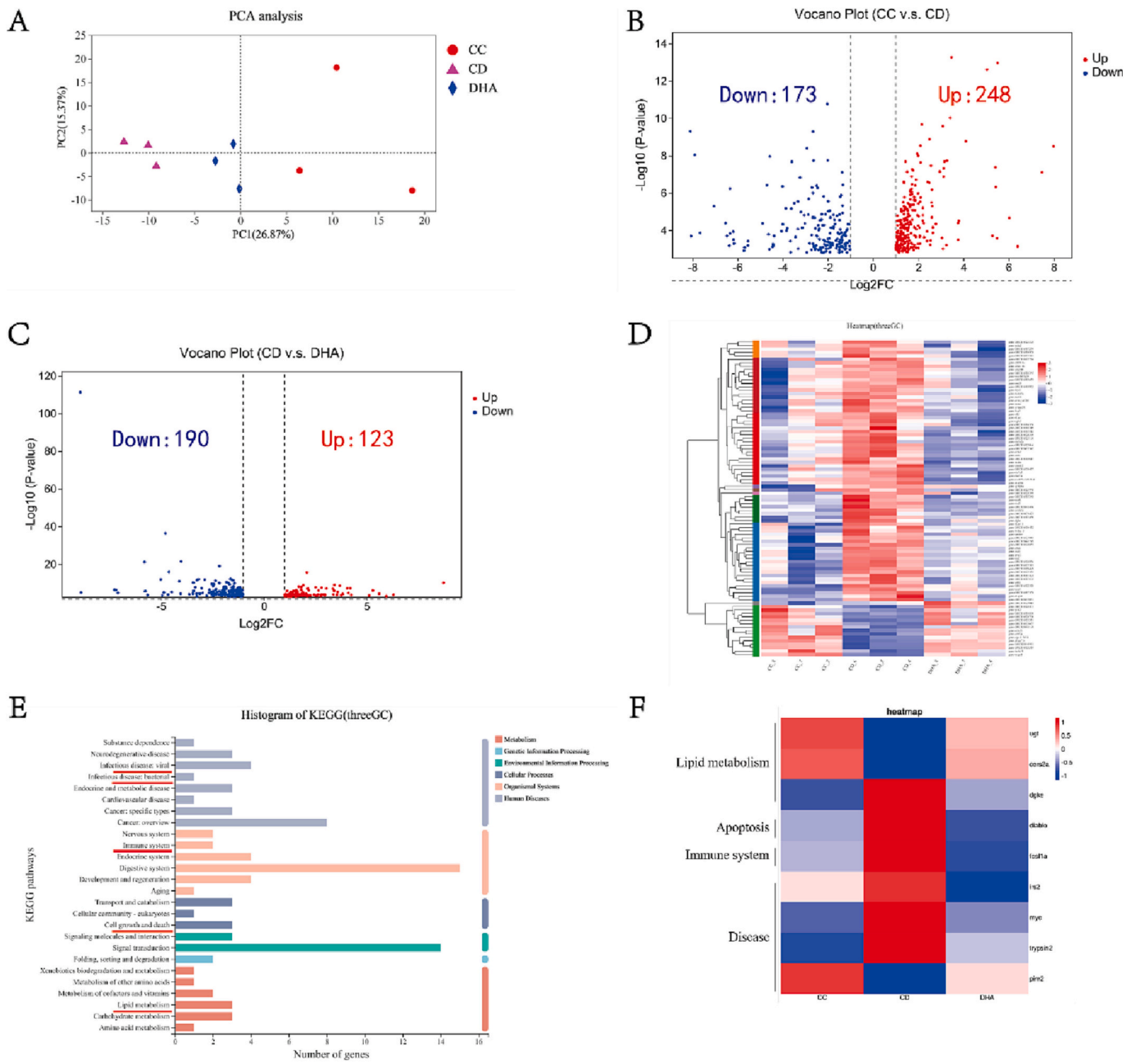


Fig. 8. Transcriptome analysis of Nile tilapia liver in CC, CD and DHA treatments. (A) Principal component analysis (PCA) in CC, CD and DHA treatments; (B) Volcano plot of differentially expressed genes (DEGs) in CC and CD treatments; (C) Volcano plot of DEGs in CD and DHA treatments; (D) Heatmap of DEGs in CC, CD and DHA treatments; and (E) Heatmap of diseases related-DEGs in CC, CD and DHA treatments.

injury in Nile tilapia. Our results showed that the expression of CerS2a in Nile tilapia liver was decreased after D-GalN/LPS injection. Interestingly, the CerS2a expression in the liver increased to a similar level as the basal condition in the DHA supplemented fish. A previous study indicated that deletion of the CerS2a cause substantial steatosis and hepatic injury in 15-month-old mice (Cao et al., 2022). Accordingly, our results suggest that, the ability of DHA to alleviate D-GalN/LPS-induced liver injury in Nile tilapia may be related to the restoration of CerS2a expression. In addition, DEGs showed that Diablo was increased in Nile tilapia liver after D-GalN/LPS injection. Diablo promotes apoptosis by antagonizing inhibitors of apoptosis proteins and activates *caspase3* and *caspase9* (Jiang et al., 2005; Mizutani et al., 2005). This is consistent with our qPCR results, suggesting that DHA alleviated Nile tilapia liver injury by inhibiting the apoptotic response. In the immune system, we found that the expression of fosl1a was up-regulated in Nile tilapia liver after D-GalN/LPS injection, while it was down-regulated in the fish supplemented with DHA. Several studies have shown that elevated fosl1a expression promotes inflammation and apoptosis (Ma et al., 2022; Yao et al., 2021; Zhong et al., 2022), consistent to our present study. We further identified four DEGs related to infectious disease, namely Irs2, Myc, Trypsin2 and Pim2. The Pim2 are biosynthetic precursors of lipoarabinomannans (Gilleron et al., 2003), which exert potent anti-inflammatory activities and strongly inhibit TLR4 signaling pathway (Doz et al., 2009). Transcriptomic DEGs results showed that PIM2 was up-regulated by DHA in Nile tilapia after D-GalN/LPS injection. This result demonstrated that the inhibition of the TLR4 signaling pathway in Nile tilapia liver by DHA is probably related to the PIM2 up-regulation. However, further researches are required to prove this hypothesis. In general, the results on transcriptomics indicate that, DHA alleviates D-GalN/LPS-induced liver injury in Nile tilapia by restoring CerS2a

expression, inhibiting apoptosis, down-regulating fosl1a and up-regulating PIM2.

5. Conclusion

In summary, our results indicated that dietary DHA supplementation reduces liver fat deposition and alleviates D-GalN/LPS-induced liver damage in Nile tilapia. It clarifies that DHA plays a protective role in Nile tilapia liver by inhibiting the TLR4 signaling pathway, reducing liver inflammation, improving liver antioxidant capacity, and reducing liver fat deposition. Our present study provides important evidence for the regulatory effect of DHA on Nile tilapia liver health for sustainable aquaculture food production.

CRediT authorship contribution statement

Y-CL, *Z*-YD conceived the study and designed the experiments. Y-CL, Z-YD, SML and J-GW carried out the experiments, analyzed the data and wrote the manuscript. Y-CL and Z-YD sampled and contributed reagents/materials. Y-CL drafted the manuscript. Y-CL, Z-YD, SML, J-GW, MW, L-QC, FQ, YL and M-LZ revised the manuscript. All authors read and approved the final manuscript.

Ethics approval

This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgements

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