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Dietary vitamin E deficiency inhibits fat metabolism, antioxidant capacity, and immune regulation of inflammatory response in genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) fingerlings following *Streptococcus iniae* infection

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ABSTRACT

Vitamin E plays an important role in maintaining normal metabolism and physiological functions in animals. The health of fish fingerlings directly affects the rate of disease incidence in adult fish, and healthy fingerlings ultimately result in better breeding outcomes for cultured fish. To date, no previous studies have focused on the effects vitamin E deficiency on tilapia at the fingerling stage. In this study, we investigated the effects of dietary vitamin E on the growth, fat metabolism, antioxidant capacity, and inflammatory response of genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) fingerlings. Vitamin E at different concentrations (0, 20, 40, 80, 160, and 320 mg/kg) was added to the diet and GIFT were fed for 55 days. Then, the GIFT were intraperitoneally injected with *Streptococcus iniae* and tested for infection. Vitamin E deficiency decreased growth and increased the food conversion ratio of GIFT fingerlings. Vitamin E deficiency also reduced the white blood cell count, increased hematocrit and hemoglobin contents in the blood, increased serum aspartate aminotransferase and alanine aminotransferase activities, and increased liver stress ($P < 0.05$). Vitamin E deficiency inhibited fat metabolism, down-regulated the expression of genes encoding lipoprotein lipase and heart-type and liver-type fatty acid-binding proteins, and increased serum total protein and fat deposition. Vitamin E deficiency significantly decreased superoxide dismutase, glutathione peroxidase, and catalase activities, increased malondialdehyde content, and caused oxidative damage. Vitamin E deficiency also up-regulated the expression of genes encoding interleukin 1β and tumor necrosis factor α in the head kidney, and stimulated a pro-inflammatory response. Overall, vitamin E deficiency inhibited growth, impaired fat metabolism, and disrupted the inflammatory response of GIFT fingerlings, whereas vitamin E supplementation in the diet reversed these negative effects. The diets with high concentrations of vitamin E (160–320 mg/kg) led to vitamin E accumulation in the fish tissues and rapid activation of the inflammatory response and antioxidant capacity in GIFT fingerlings exposed to *S. iniae*.

1. Introduction

When aquatic animals are exposed to environmental stress, their physiological state changes triggering a series of response reactions involving the nervous, endocrine, and immune systems. These responses result in low immunity and resistance, which can affect growth and development, and even cause death in severe cases [1]. Therefore, improving the immune function of aquatic animals is conducive to healthy growth and reduced mortality. Vitamins are micro-nutrients required for normal growth, reproduction, metabolism, and immune function [2].

Vitamin E is a fat-soluble vitamin that has two forms, tocopherols and tocotrienols. Aquatic animals cannot synthesize vitamin E, so it must be provided in their diet. Vitamin E is widely involved in antioxidant, cell signaling, reproductive development, immune regulation, and anti-stress processes [3,4]. It is a potent antioxidant that protects

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against oxidative damage in various fish tissues [5], enhances resistance of red blood cell (RBC) membranes [6], and protects leukocyte functions [7]. In previous studies, appropriate dietary supplementation with vitamin E significantly increased the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in *Penaeus monodon* [8] and *Litopenaeus vannamei* [9], and reduced the malondialdehyde (MDA) content. Palace et al. [10] found that a low concentration of vitamin E in the diet enhanced SOD activity in rainbow trout (*Oncorhynchus mykiss*). Pan et al. [11] showed that vitamin E deficiency caused oxidative damage in the head kidney, spleen, and skin of grass carp (*Ctenopharyngodon idella*), and this damage was associated with low activities of SOD, GSH-Px, CAT, and glutathione reductase. Adding vitamin E to the diet of genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) juveniles reduced the MDA content in the liver and decreased oxidative stress [12]. Different fish species may show different physiological effects of vitamin E deficiency. Vitamin E has been shown to play an important role in preventing lipid peroxidation to protect the integrity of tissues in various fishes, including turbot (*Scophthalmus maximus*), gilthead sea bream (*Sparus aurata*) [13], Atlantic halibut (*Hippoglossus hippoglossus*) [14], and black sea bream (*Acanthopagrus schlegelii*) [15]. Vitamin E also can regulate stress responses produced by microcysts [16], heavy metal pollution [17], crowd stress [18], oxidized fish oil [19], and high-fat diets [20].

The liver is the main site for lipid metabolism in fish. The normal operation of lipid metabolism depends on many related factors, including lipoprotein lipase (LPL) and fatty acid binding proteins (FABPs). LPL lipolyzes the triglycerides in very low density lipoproteins to produce glycerol and fatty acids; while FABPs transport long-chain fatty acids and participate in fat transport processes that regulate intracellular fatty acid concentration and mediate fatty acid metabolism [21,22]. Vitamin E is considered an essential regulatory factor for fat metabolism. Little is known about the role of vitamin E in fat metabolism in aquatic animals because most previous studies on fat metabolism have focused on land animals. For example, the level of vitamin E supplementation in the diet of Guangxi Sanhuang chicken was found to be significantly positively correlated with the expression of genes encoding LPL and FABPs in the liver, which affected lipid metabolism [23]. A high dose of vitamin E (1600 mg/kg) in diet of rats inhibited their growth, and reduced the synthesis of very high density lipoprotein, triglycerides, and cholesterol *in vivo*, which negatively affected lipid metabolism [24]. As the level of fat in the diet of fish increases, the amount of vitamin E required also increases [20], but the reason for this is still unknown. One aim of the present study was to investigate this point further.

Previous studies have demonstrated that the dietary lipid content and lipid source significantly affect lipid metabolism and liver fat deposition. Abnormal fat deposition and metabolic disorders may increase the level of pro-inflammatory factors in the liver, leading to an inflammatory reaction that negatively affects the animal's immunity [25,26]. Mu et al. [26] found that high levels of soybean oil supplementation significantly reduced the expression of genes encoding anti-inflammatory cytokines (arginase I and interleukin 10) and increased the expression of genes encoding pro-inflammatory cytokines (tumor necrosis factor α and interleukin1β) in large yellow croaker (*Larimichthys crocea*). Another focus of this study was to determine whether vitamin E deficiency affects the inflammatory response of fish by regulating hepatic fat metabolism.

Genetically improved farmed tilapia is one of the world's major farmed fish. Recently, high-density culture, aquaculture water pollution, and unbalanced feed nutrition has led to greatly increased incidence of *Streptococcus* infections in GIFT. Infected individuals are found from the adult fish stage to the fingerling stage. Improving the health status at the fingerling stage through nutritional intervention may help to reduce the risk of *Streptococcus* infections in GIFT at all stages of growth and development.

The aims of this study were as follows: 1) to determine the optimum amount of vitamin E supplementation at the GIFT fingerling stage; 2) to determine the effect of vitamin E deficiency on lipid metabolism of GIFT fingerlings; and 3) to investigate the effect of vitamin E deficiency on antioxidant activity and the inflammatory response of GIFT fingerlings and the regulatory mechanism involved in the response to *Streptococcus iniae* infection. The overall aim of this study was provide a partial theoretical basis for the role of vitamin E in regulating lipid metabolism and the inflammatory response in GIFT fingerlings. The requirement for vitamin E in the diet of GIFT fingerlings also was evaluated to provide a reference for the preparation of commercial feed.

2. Material and methods

2.1. Experimental system and GIFT fingerling rearing

We used a closed freshwater recirculating aquaculture system with a water flow rate of 3 L/min and continuous aeration supplied by compressed air and air stones. The system was located at the Greenhouse Facility of the Key Laboratory for Genetic Breeding of Aquatic Animals and Aquaculture Biology, Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fishery Sciences, Wuxi, China.

The GIFT fingerlings were obtained from the Yixing tilapia base of the FFRC and transported in oxygenated plastic tanks. Upon arrival, the fingerlings were stocked in plastic tanks and fed to satiation three times daily with a commercial diet for 1 week of acclimatization. After acclimatization, the fish were starved for 24 h and batch-weighed using an electronic scale balance. The fish with mean weight of 0.66 ± 0.02 g were selected and randomly assigned into six groups with four replicates. The tanks were filled with 800 L dechlorinated freshwater at a stocking density of 60 fish per tank. A total of 1440 fingerlings were used in this study. Mortalities were recorded and replacements were made within the first week of the experiment. The fish were fed three times daily close to satiation at 08:00, 13:00, and 17:00 for 55 days. Uneaten feed and fecal matter were removed daily. The following conditions were maintained: water temperature 26–28 °C; dissolved oxygen concentration > 5.0 mg/L; and light/dark photoperiod of 12 h/12 h.

2.2. Experimental diet preparation

Vitamin E (DL-α-tocopherol acetate ≥96%; Sigma Chemical Co. Steinheim, Germany) was added at different concentrations to a basal diet to formulate diets containing 0, 20, 40, 80, 160, and 320 mg vitamin E/kg. The diets were prepared by thoroughly mixing the powdered raw materials (ingredients) and then adding oils and water until a stiff dough was obtained. The dough was passed through a laboratory pelletizer resulting in strands of feed that were gently broken into pellets while fresh. The pellets (crude protein 33.4%; crude fat 7.3%) were then air dried, packed into airtight plastic bags, and stored at 4 °C until use (see Table 1).

2.3. Sample collection and management

At the end of the 55-day experiment, the fish were starved for 24 h to empty the gastrointestinal tract. Six fish per tank were selected randomly and each weighed using an electronic scale. Throughout the experiment, the amount of feed consumed and mortality in each replicate were recorded.

Fish growth was determined by calculating the final body weight, specific growth rate, and hepatosomatic index. Feed utilization was determined by calculating the food conversion ratio. Survival was expressed as a percentage. The calculations for these indexes are as follows:

- Specific growth rate (SGR, %/d) = (In FBW − In IBW)/T × 100

Table 1
Feed ingredients.

Ingredient/Group	1	2	3	4	5	6
Vitamin E(mg/kg)	0	20	40	80	160	320
Soybean meal	20	20	20	20	20	20
Casein	22	22	22	22	22	22
Gelatin	5.5	5.5	5.5	5.5	5.5	5.5
Soybean oil	7	7	7	7	7	7
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5
VC phosphate sodium	0.2	0.2	0.2	0.2	0.2	0.2
$Ca(H_2PO_4)_2$	1.5	1.5	1.5	1.5	1.5	1.5
Removal of VE multivitamins[a]	1	1	1	1	1	1
Composite mineral salt[b]	1	1	1	1	1	1
Dextrin	31.5	31.5	31.5	31.5	31.5	31.5
Cellulose	9.8	9.8	9.8	9.8	9.8	9.8
Total	100	100	100	100	100	100

[a] Vitamin premix (mg/kg dry diet): V_A 10, V_D 0.05, V_K 40, V_{B1} 50, V_{B2} 200,V_{B3} 500, V_{B6} 50, V_{B7} 5, V_{B11} 15, V_{B12} 0.1,V_C 1000, inositol 2000, choline 5000.

[b] Mineral premix (mg/kg dry diet): $FeSO_4{\cdot}7H_2O$ 372, $CuSO_4{\cdot}5H_2O$ 25, $ZnSO_4{\cdot}7H_2O$ 120, $MnSO_4{\cdot}H_2O$ 5, $MgSO_4$ 2475, NaCl 1875, KH_2PO_4 1000, $Ca(H_2PO_4)_2$ 2500.

where, FBW = final body weight (g); IBW = initial body weight (g); and T is the number of test days.

- Feed conversion ratio (FCR) = Feed intake/wet weight gained
- Cumulative survival (%) = (number of fish harvested/number of fish stocked) × 100
- Hepatosomatic index (HSI, %) = (liver weight/whole body weight) × 100.

Three fish were selected randomly per tank and blood samples were taken from the caudal vein using a sterile 1-ml syringe after sedating the fish with 200 mg/L MS-222 (tricaine methanesulfonate). The syringes were not rinsed with anticoagulant, but it took less than 10 s to draw blood from each fish, so no coagulation occurred. About 1 ml blood was taken from each fish. The collected blood samples were each divided into two portions. One portion (20 μl) was transferred into a 1.5-ml labeled sterile Eppendorf tube containing 100 μl phosphate buffer saline (PBS, 0.02 M, pH 7.3) solution as a diluent for the hematological assay. The other portion was kept at 4 °C for 3 h, then centrifuged at 5000 g for 15 min at 4 °C to obtain serum. The serum was immediately collected into another labeled tube and stored at −80 °C until analysis of biochemical indices.

The three sampled fish from each tank were dissected and the liver, intestine, and head kidney were removed, immediately frozen in liquid nitrogen and preserved at −80 °C. The intestine and liver samples were used for analysis of related enzyme activities. The head kidney samples were used for RNA extraction.

2.4. S. iniae infection

To reduce sampling stress, each experimental group was raised for another 10 days as described in section 2.1. Culture and counting of *S. iniae* were carried out as described by Qiang et al. [27]. The bacterium used in this experiment was a *S. iniae* culture preserved in our laboratory. To rejuvenate the *S. iniae* strain, the bacterium was cultured in brain heart infusion medium in a shaking incubator at 28 °C for 24 h. The culture was centrifuged at 4 000 g for 15 min to collect bacteria, which were then washed with sterilized physiological saline (0.85%). The bacterial pellet was diluted by 10 times with 0.85% physiological saline solution. A preliminary experiment was performed prior to the main experiment to determine the appropriate concentration of the bacterial challenge. The semi-lethal concentration of bacterial infection was roughly estimated by the turbidimetric method. In the normal experiments, the concentration of the bacterial solution was calculated by the dilution plate counting method. The concentration of *S. iniae* in the injection was 5.8×10^5 CFU/ml. Ten fish were selected randomly from each tank to calculate the cumulative survival rate against *S. iniae* infection. Another 25 fish were selected after infection for the sampling experiment. Three GIFT were sampled randomly from each tank at 24, 48, and 96 h post-infection. Liver and head kidney samples were collected, immediately frozen in liquid nitrogen, and preserved at −80 °C until further analysis.

2.5. Measurement of parameters

2.5.1. Blood parameters

The total white blood cell count (WBC, 10^9/L) was determined in a 1:100 dilution of each blood sample in PBS solution, and the RBC (10^{12}/L) was determined in a 1:1000 dilution of each blood sample. The blood cells were counted with a Neubauer hemocytometer as described in our earlier studies [27,28]. The hematocrit percentage (Ht, %) was determined using the microhematocrit method (Micro-hematocrit centrifuge, 346, UNIPAA, Poland), with duplicate samples centrifuged for 10 min [29]. The hemoglobin (Hb, g/L) concentration was calculated using the cyanomethemoglobin method, from the absorbance value measured at 540 nm [30].

2.5.2. Serum parameters

The concentrations of total protein (TP), triglyceride (TG), total cholesterol (TC), and glucose, and the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using the serum samples. All these indices were measured using an automatic biochemical analyzer (Mindray BS-400; Mindray, Shenzhen, China). All test kits were purchased from Shenzhen Mindray Bio-Medical Electronics Co. Ltd (Shenzhen, China).

2.5.3. Liver parameters

The liver samples (0.9–1.00 g) were weighed and homogenized in ice-cold PBS, then centrifuged for 15 min at 5000 g at 4 °C and stored at −40 °C until analysis of related enzymes. The protein concentration in the supernatant was determined by the Coomassie brilliant blue assay. The activities of GSH-Px, SOD, and CAT, and the MDA content were determined using kits purchased from the Nanjing Institute of Bioengineering (Nanjing, China), according to the manufacturer's instructions. The specific test methods have been described in detail by He et al. [31].

2.5.4. Intestinal digestive enzyme activities

Protease activity [μg/(g·min] was determined by the Folinphenol reagent method, and reflects the amount of tyrosine (μg) produced by hydrolysis of casein per min from 1 g fresh tissue at 28 °C and pH 7.4 [32]. Amylase activity was determined by the hypoiodic acid method. Amylase activity [mg/(g·min)] represents the amount of glucose (mg) produced by the catalytic decomposition of starch per min from 1 g fresh tissue at 28 °C and pH 7.4 [32]. Lipase activity was determined by hydrolysis of polyvinyl alcohol olive oil emulsion. Lipase activity [μg/(g·min)] reflects the amount of fatty acid (μg) produced per min by catalysis of a polyvinyl alcohol olive oil emulsion from 1 fresh tissue at 28 °C and pH 7.4 [33]. All kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.5.5. Vitamin E contents in tissues

A 0.1 g portion of liver or intestine sample was added to 0.86% physiological saline (weight (g): volume (ml) = 1:9), fully homogenized on ice, and then centrifuged at 2500 g for 10 min. The supernatant was used to determine the vitamin E content of the sample by high performance liquid chromatography (JY/T 024–1996).

2.5.6. Gene expression analyses

The nucleotide sequences of genes encoding tilapia interleukin 1β

Table 2
Primer sequences of qRT-PCR.

Name	Primer sequence (5′-3′)
IL-1β	F: 5′- CAAGGATGACGACAAGCCAACC-3′
	R: 5′- AGCGGACAGACATGAGAGTGC-3′
TNF-α	F: 5′- GGAAGCAGCTCCACTCTGATGA-3′
	R: 5′- CACAGCGTGTCTCCTTCGTTCA-3′
IFN-γ	F: 5′- AAGAATCGCAGCTCTGCACCAT-3′
	R: 5′- GTGTCGTATTGCTGTGGCTTCC-3′
LPL	F: 5′- ATCAGCACTACCCGACCTCT-3′
	R: 5′- GCGCTCCCAGACTATAACCC-3′
H-FABP	F: 5′- CAACCGGCTCAAATGCCATC-3′
	R: 5′- GTTTGCATCCACCAGTTCCG-3′
L-FABP	F: 5′-TTCGAAGACATCCACGCAGT-3′
	R: 5′-AGTTTTGGGAGGCTGTCACT-3′
I-FABP	F: 5′-ACCGCCACCATGACTTTCAA-3′
	R: 5′-GGTCCACGCACCTGATAGTT-3′
18S rRNA	F: 5′-GGCCGTTCTTAGTTGGTGGA-3′
	R: 5′-TTGCTCAATCTCGTGTGGCT-3′

IL-1β: Interleukin 1β; TNF-α: tumor necrosis factor α; IFN-γ: Interferon γ; LPL: lipoprotein lipase; H-FABP: heart-type fatty acid binding protein; L-FABP: liver-type fatty acid binding protein; I-FABP: intestine-type fatty acid binding protein.

(IL-1β), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), LPL, heart-type FABP (H-FABP), liver-type FABP (L-FABP), and intestinal-type FABP (I-FABP) were obtained from GenBank and used to design specific primers for these genes. Tilapia *18S rRNA* was used as the reference gene. The primer sequences are listed in Table 2. All the primers were synthesized by the Shanghai Jikang Biotechnology Co., Ltd (Shanghai, China). Total RNA was extracted from about 20 mg head kidney or liver samples. The OD260/OD280 was about 1.90. The sample preparation and reaction conditions for quantitative reverse transcription PCR analyses (qRT-PCRs) have been described in detail by Qiang et al. [28].

2.6. Data analyses

The relative transcript levels of the mRNAs were calculated assuming that mRNA expression was substantially consistent with the qRT-PCR amplification efficiency of the tilapia *18S rRNA* reference gene. The Ct value was calculated for each sample and normalized. Based on the lowest relative expression value, the relative transcript level of each gene in fish in each treatment was determined by the $2^{-\Delta\Delta CT}$ method. The results are expressed as mean ± standard deviation (mean ± SD). The data were tested for normality of distribution and homogeneity of variances before further analyses. The relationship between SGR and the dietary vitamin E levels was analyzed by the two-slope broken-line model (Fig. 1). The significance level was $P \leq 0.05$.

3. Results

3.1. Growth performance, survival, and feed utilization

The highest FBW was in the group supplemented with 80 mg/kg vitamin E and the lowest was in the control group (0 mg vitamin E) (Table 3). The FBW differed significantly among the different treatments ($P < 0.05$). The trend in SGR was similar to that of FBW and was within the range of 6.94%/d to 7.23%/d. The lowest FCRs recorded were in the 80 and 160 mg/kg treatment groups, and the highest FCR was in the control group. The HSI did not differ significantly ($P > 0.05$) among all groups (0–320 mg/kg vitamin E supplementation). In the 80–160 mg/kg treatment groups, the HSI values (1.42–1.43) were slightly higher than in the control group (1.34); however, the differences were not significant. As shown in Table 3, the α-tocopherol contents in the liver and intestine were higher in the 20–320 mg/kg vitamin E treatment groups than in the control group

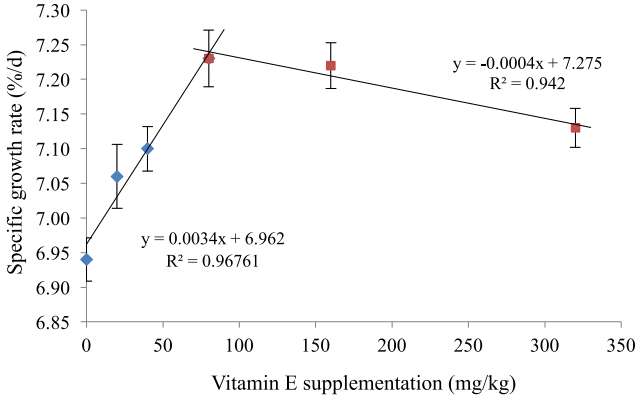

Fig. 1. The relationship between specific growth rate and the dietary vitamin E levels was analyzed by two slope broken-line model.

($P < 0.05$).

3.2. Hematological indices

The WBC count differed significantly between vitamin E-deficient GIFT fingerlings and those fed a diet supplemented with 80–320 mg/kg vitamin E ($P < 0.05$), but did not differ significantly ($P > 0.05$) among the 40, 80, 160, and 320 mg/kg treatment groups (Table 4). The RBC did not differ significantly ($P > 0.05$) among the 0–320 mg/kg dietary vitamin E-treated groups. The Hb and Ht% differed significantly ($P < 0.05$) between the 320 mg/kg and control groups, but did not differ significantly ($P > 0.05$) among the other four treatment groups (20, 40, 80, and 160 mg/kg vitamin E).

3.3. Serum biochemical parameters

The TP contents were significantly lower in the 0–160 mg/kg vitamin E treatment groups than in the 320 mg/kg vitamin E treatment group ($P < 0.05$) (Table 5). Glucose was significantly elevated in the 40 and 80 mg/kg groups compared with the control group, but glucose content did not differ significantly ($P > 0.05$) among the four treatment groups (40, 80, 160, and 320 mg/kg). The TC and TG levels were significantly reduced in the 80 and 160 mg/kg vitamin E treatment groups compared with the control group. The ALT and AST activities were significantly decreased ($P < 0.05$) in the 40 and 80 mg/kg groups compared the control group and the 320 mg/kg treatment group, but did not differ significantly ($P > 0.05$) between the control group and the 320 mg/kg treatment group.

3.4. Digestive enzymes in intestine

Trypsin activity in the intestine was significantly increased ($P < 0.05$) in GIFT fingerlings fed diets containing vitamin E (Table 6). Amylase activity did not differ significantly ($P > 0.05$) among the different treatment groups. Lipase activity in the intestine was significantly higher in the 20–320 mg/kg treatment groups than in the control group; but did not differ significantly ($P > 0.05$) among the 20–320 mg/kg treatment groups.

3.5. Expression levels of lipid metabolic enzyme genes in liver

As shown in Fig. 2, the transcript levels of *LPL*, *H-FABP*, and *L-FABP* in the liver of GIFT fingerlings increased with increasing levels of vitamin E supplementation in the diet. The transcript level of *LPL* was significantly increased ($P < 0.05$) in the 40, 80, 160, and 320 mg/kg groups compared with the control group. The transcript levels of *H-FABP* and *L-FABP* did not differ significantly among the 40–160 mg/kg

Table 3
Effect of dietary vitamin E levels on growth performance and α-tocopherol content of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*).

Vitamin E (mg/kg)	0	20	40	80	160	320
IBW	0.65 ± 0.03	0.66 ± 0.02	0.65 ± 0.03	0.66 ± 0.03	0.66 ± 0.02	0.66 ± 0.03
FBW	31.53 ± 0.71^{a}	34.33 ± 0.88^{ab}	35.20 ± 0.56^{ab}	37.81 ± 0.82^{b}	37.74 ± 0.79^{b}	35.98 ± 0.93^{ab}
SGR (%/d)	6.94 ± 0.31^{a}	7.06 ± 0.46 ^{ab}	7.10 ± 0.32 ^{ab}	7.23 ± 0.41^{b}	7.22 ± 0.33^{b}	7.13 ± 0.28 ^{ab}
FCR	1.62 ± 0.04^{b}	1.53 ± 0.06 ^{ab}	1.51 ± 0.09 ^{ab}	1.47 ± 0.05^{a}	1.47 ± 0.03^{a}	1.53 ± 0.01^{ab}
HSI	1.34 ± 0.13	1.32 ± 0.02	1.43 ± 0.14	1.42 ± 0.06	1.43 ± 0.05	1.34 ± 0.22
S	95.00 ± 3.72	94.17 ± 4.93	96.67 ± 2.36	95.83 ± 2.76	95.83 ± 3.63	95.83 ± 1.44
α-tocopherol in liver	14.15 ± 1.44^{a}	25.75 ± 1.78^{b}	56.50 ± 3.54^{c}	107.62 ± 9.76^{d}	166.94 ± 11.73^{e}	237.29 ± 12.85^{f}
α-tocopherol in intestine	3.84 ± 0.39^{a}	13.81 ± 1.43^{b}	14.01 ± 1.31^{b}	34.23 ± 1.37^{c}	74.64 ± 4.56^{d}	95.00 ± 6.44^{e}

Differences between the two groups were compared using an independent sample T test, and were considered significant at $P < 0.05$. Different lowercase letters show significant differences among different treatment groups.
IBW: initial body weight; FBW: final body weight; SGR: specific growth rate; FCR: feed conversion ratio; HSI: hepatosomatic index; S: survival.

groups, but the transcript level of *H-FABP* was significantly higher in the 320 mg/kg group than in the 20 and 40 mg/kg groups. The transcript levels of *I-FABP* did not differ significantly among the different treatment groups ($P > 0.05$).

3.6. Cumulative mortality in S. iniae-infected GIFT fingerlings in different treatment groups

The highest cumulative survival was recorded for fish fed the diets containing 320 and 160 mg/kg vitamin E, followed by those fed the diets containing 80 and 40 mg/kg vitamin E (Fig. 3). The lowest cumulative survival was in the control group, followed by the group fed the diet containing 20 mg/kg vitamin E. The cumulative survival rates differed significantly among the different treatment groups ($P < 0.05$).

3.7. Activities of hepatic antioxidative enzymes in GIFT fingerlings in different treatment groups pre- and post-S. iniae infection

At the end of the trial, SOD activity was significantly higher ($P < 0.05$) in the vitamin E treatment groups than in the control group pre-*S. iniae* infection (Fig. 4A). The SOD activity did not differ significantly among the 20–160 mg/kg groups, but was significantly higher in the 320 mg/kg group than in the other treatment groups. Post-*S. iniae* infection, SOD activity was significantly higher in all the treatment groups than in the control group. At 24 h, the SOD activity in the liver of the fingerlings was significantly higher in the 80 mg/kg group than in the control group, but not significantly different among the 20 and 40 mg/kg treatment groups and the control group ($P > 0.05$). At 48 h and 96 h, the SOD activity was significantly higher in the 160 and 320 mg/kg groups than in the control group. At 96 h, no significant differences were detected among the 20, 40, and 80 mg/kg treatment groups and the control group.

The CAT and GSH-Px activities differed significantly between the 160 mg/kg group ($P < 0.05$) and the control group and the 320 mg/kg group pre-*S.iniae* infection (Fig. 4B, D). The CAT and GSH-Px activities tended to be higher in all the treatment groups than in the control group at 48 h post-*S.iniae* infection. The CAT and GSH-Px activities in the liver of the fingerlings were significantly higher ($P < 0.05$) in the 80 and 160 mg/kg groups than in the control group and the 20 mg/kg treatment group. At 96 h, no significant differences in CAT activity were detected among the treatment groups ($P > 0.05$), whereas GSH-Px activity was significantly higher in the 80 and 160 mg/kg groups than in the control and 20 mg/kg groups ($P < 0.05$).

Pre-*S.iniae* infection, hepatic MDA levels in the 80 and 160 mg/kg groups differed significantly ($P < 0.05$) from those in the control and 320 mg/kg groups (Fig. 4C), and MDA levels tended to be lower in the 20–160 mg/kg groups. Post-*S.iniae* infection, the MDA levels were significantly increased in all treatment groups. At 48 h, the MDA levels were significantly higher in the control, 20, and 40 mg/kg groups than in the 80 and 160 mg/kg groups. At 96 h, the MDA level was significantly higher in the control group than in the treatment groups ($P < 0.05$).

3.8. Inflammatory response in head kidney of GIFT fingerlings fed different amounts of dietary vitamin E pre- and post-S. iniae infection

At the end of the trial, the transcript levels of *IL-1β* and *TNF-α* in the head kidney of GIFT fingerlings were significantly lower in the 80–320 mg/kg treatment groups than in the control group pre-*S.iniae* infection (Fig. 5A and B). However, the transcript level of *IFN-γ* mRNA in the head kidney did not differ significantly among the treatment groups (Fig. 5C). An agarose gel electrophoresis analysis showed no significant differences in the abundance of *18S rRNA* among the treatment groups at 96 h post-*S.iniae* infection, confirming that *18S rRNA* was a reliable reference gene. The transcript levels of *IL-1β, TNF-α,* and *IFN-γ* in all treatment groups first increased and then decreased. Their transcript levels were highest at 48 h and decreased significantly at 96 h post-*S.iniae* infection. Compared with the control group, the 40–320 mg/kg groups showed significantly increased transcript levels of pro-inflammatory genes in the head kidney at 48 h and 96 h post-*S.iniae* infection ($P < 0.05$).

4. Discussion

Vitamin E added to aquatic animal feed can effectively prevent damage to body tissues caused by peroxidation of unsaturated fatty acids in the diet and body tissues, and help to improve protein absorption and growth performance. In this study, the GIFT fingerlings in

Table 4
Effect of dietary vitamin E levels on blood parameters of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*).

Vitamin E (mg/kg)	0	20	40	80	160	320
WBC (10^9/L)	203.19 ± 5.79^{a}	208.07 ± 4.57^{a}	227.32 ± 7.97^{ab}	229.72 ± 9.11^{b}	239.45 ± 10.23^{b}	233.25 ± 6.42^{b}
RBC (10^{12}/L)	2.36 ± 0.08	2.43 ± 0.06	2.54 ± 0.12	2.61 ± 0.16	2.36 ± 0.15	2.25 ± 0.10
HGB (g/L)	141.17 ± 5.27^{b}	130.08 ± 3.08^{ab}	133.08 ± 6.09^{ab}	135.25 ± 7.95^{b}	123.75 ± 7.74^{ab}	119.75 ± 5.78^{a}
HCT (%)	41.93 ± 1.32^{c}	39.38 ± 1.11^{bc}	39.43 ± 1.75^{bc}	39.40 ± 3.78^{bc}	38.14 ± 2.47^{ab}	36.59 ± 1.90^{a}

Differences between the two groups were compared using an independent sample T test, and were considered significant at $P < 0.05$. Different lowercase letters show significant differences among different treatment groups.
WBC: white blood cell count; RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit.

Table 5
Effect of dietary vitamin E levels on serum biochemical parameters of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*).

Vitamin E (mg/kg)	0	20	40	80	160	320
TP(g/L)	9.26 ± 0.11^{bc}	9.26 ± 0.12^{bc}	9.34 ± 0.13^{bc}	8.78 ± 0.39^{b}	7.85 ± 0.53^{a}	9.92 ± 0.40^{c}
Glucose(mmol/L)	0.73 ± 0.04^{a}	0.80 ± 0.03^{ab}	0.87 ± 0.04^{b}	0.91 ± 0.08^{b}	0.84 ± 0.08^{ab}	0.92 ± 0.06^{b}
TC(g/L)	1.13 ± 0.06^{b}	1.16 ± 0.05^{b}	1.11 ± 0.05^{b}	1.07 ± 0.07^{ab}	1.00 ± 0.06^{a}	1.17 ± 0.05^{b}
TG(g/L)	0.59 ± 0.10^{c}	0.59 ± 0.14^{c}	0.39 ± 0.04^{bc}	0.31 ± 0.04^{ab}	0.24 ± 0.03^{a}	0.32 ± 0.03^{ab}
ALT(U/L)	4.73 ± 0.27^{b}	3.82 ± 0.33^{a}	4.05 ± 0.4^{a}	4.07 ± 0.31^{a}	4.12 ± 0.42^{a}	4.53 ± 0.34^{b}
AST(U/L)	24.14 ± 1.46^{b}	25.67 ± 1.28^{b}	19.34 ± 1.04^{a}	18.22 ± 1.25^{a}	20.17 ± 2.11^{ab}	23.12 ± 2.01^{b}

Differences between the two groups were compared using an independent sample T test, and were considered significant at $P < 0.05$. Different lowercase letters show significant differences among different treatment groups.
TP: total protein; TC: total cholesterol; TG: triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

the control (vitamin E deficiency) group grew slowly and showed a low feed efficiency, while those fed the diets supplemented with vitamin E showed significantly improved growth. The broken-line regression analysis of the FCR showed that 92.06 mg/kg vitamin E was the optimal amount for GIFT fingerlings to achieve optimal growth. This is higher than the optimal amount of vitamin E (10–25 mg/kg) determined for blue tilapia, *Oreochromis aureus* [34], and the recommended amount of vitamin E (50 mg/kg) for adult tilapia [35]. The requirement determined for GIFT fingerlings is also higher than those determined for yellow catfish (*Pelteobagrus fulvidraco*) (39.7 mg/kg) [36], hybrid striped bass (*Morone chrysops*♀ × *M. saxatilis*♂) (28 mg/kg) [37], and Korean rockfish (*Sebastes schlegelii*) (45 mg/kg) [38], but similar to that reported for grass carp (*Ctenopharyngodon idella*) (90–135 mg/kg) [11]. These differences may be related to the fish species, the composition and palatability of the feed, the size of the fish, feed frequency and level, and the experimental conditions. In the 320 mg/kg vitamin E treatment group, the SGR showed a downward trend and the FCR showed an upward trend. High doses of vitamin E can decrease in utilization of other fat-soluble vitamins such as vitamins D and K [39], and excess vitamin E may be used to improve immunity rather than growth.

We found that vitamin E deficiency in the diet significantly increased the amount of Hb and Ht% in the blood of the GIFT fingerlings. The changes in Hb level and Ht% may be related to the nutritional status of the fish. A lack of vitamin E may inhibit the fishes' utilization and reaction rate of dietary fat, which might increase the metabolic burden. In that case, the fish would need more oxygen to transport fat in the blood, resulting in an increase in RBC and Hb levels [40]. In previous studies, appropriate vitamin E supplementation decreased the metabolic energy demand of sturgeon (*Acipenser naccarii*) [41] and gilthead seabream (*Sparus aurata*) [42]. In this study, the higher blood WBC levels detected in the high-dose vitamin E-supplemented groups (160 and 320 mg/kg) may be associated with better non-specific immunity in these fishes.

Serum biochemical parameters are considered to have a significant impact on the overall health and immunity of animals; therefore, the serum levels of such substances need to be well balanced to maintain good health of cultured organisms. Andrade et al. [43] found that high concentrations of vitamins C and E could stimulate protein production in fish, implying the important roles of these vitamins in modulating plasma proteins. Similarly, we found that high doses of vitamin E or vitamin E deficiency significantly increased the TP content in the

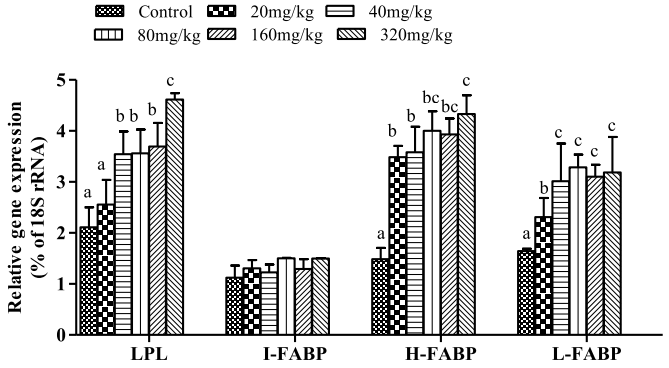

Fig. 2. Effects of dietary vitamin E levels on the lipid metabolism related gene expressions of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*). LPL: lipoprotein lipase; L-FABP: liver-type fatty acid binding protein; H-FABP: heart-type fatty acid binding protein; I-FABP: intestine-type fatty acid binding protein.

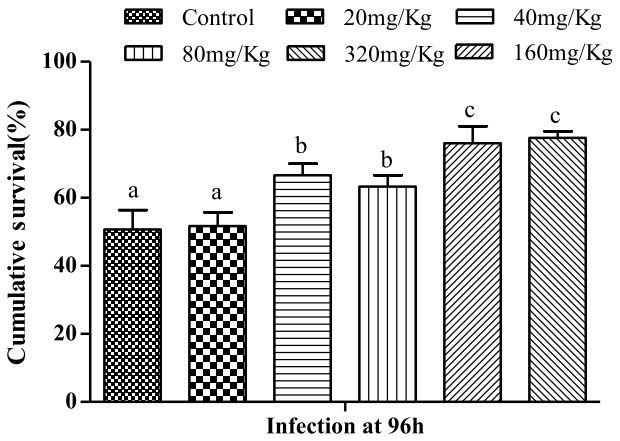

Fig. 3. Effects of dietary vitamin E levels on the cumulative survival of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*) at 96 h post- *Streptococcus iniae* infection.

Table 6
Effect of dietary vitamin E levels on intestinal digestive enzymes of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*).

Vitamin E (mg/kg)	0	20	40	80	160	320
Trypsin (U/mL)	31.11 ± 2.13^{a}	39.56 ± 3.24^{b}	43.52 ± 3.25^{c}	48.92 ± 3.57^{d}	39.24 ± 2.17^{b}	39.71 ± 4.16^{b}
Amylase (U/mL)	83.62 ± 6.15	84.17 ± 7.21	87.21 ± 6.54	85.91 ± 5.72	86.43 ± 6.58	85.14 ± 6.26
Lipase (U/mL)	101.32 ± 8.36^{a}	116.52 ± 7.78^{b}	112.35 ± 8.11^{ab}	117.25 ± 9.03^{b}	121.11 ± 8.34^{b}	118.92 ± 9.43^{b}

Differences between the two groups were compared using an independent sample T test, and were considered significant at $P < 0.05$. Different lowercase letters show significant differences among different treatment groups.

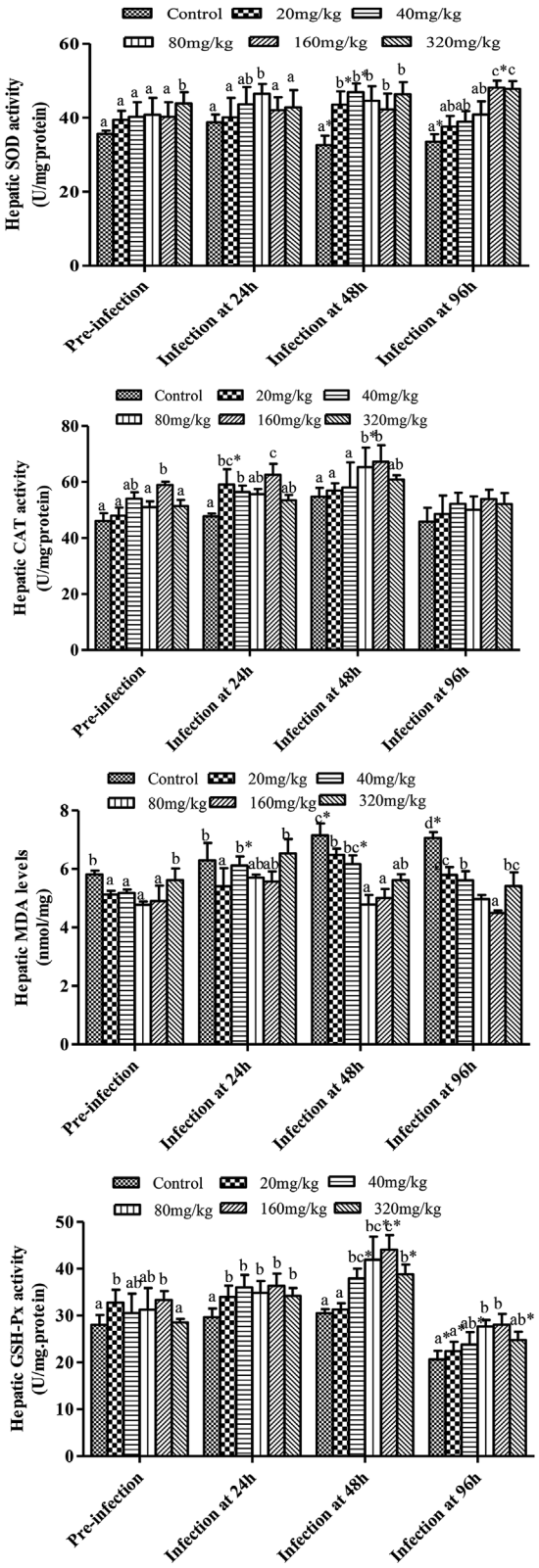

Fig. 4. Effects of dietary vitamin E levels on the activities of hepatic antioxidative enzymes of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*) pre- and post- *Streptococcus iniae* infection. SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase; MDA: malondialdehyde.

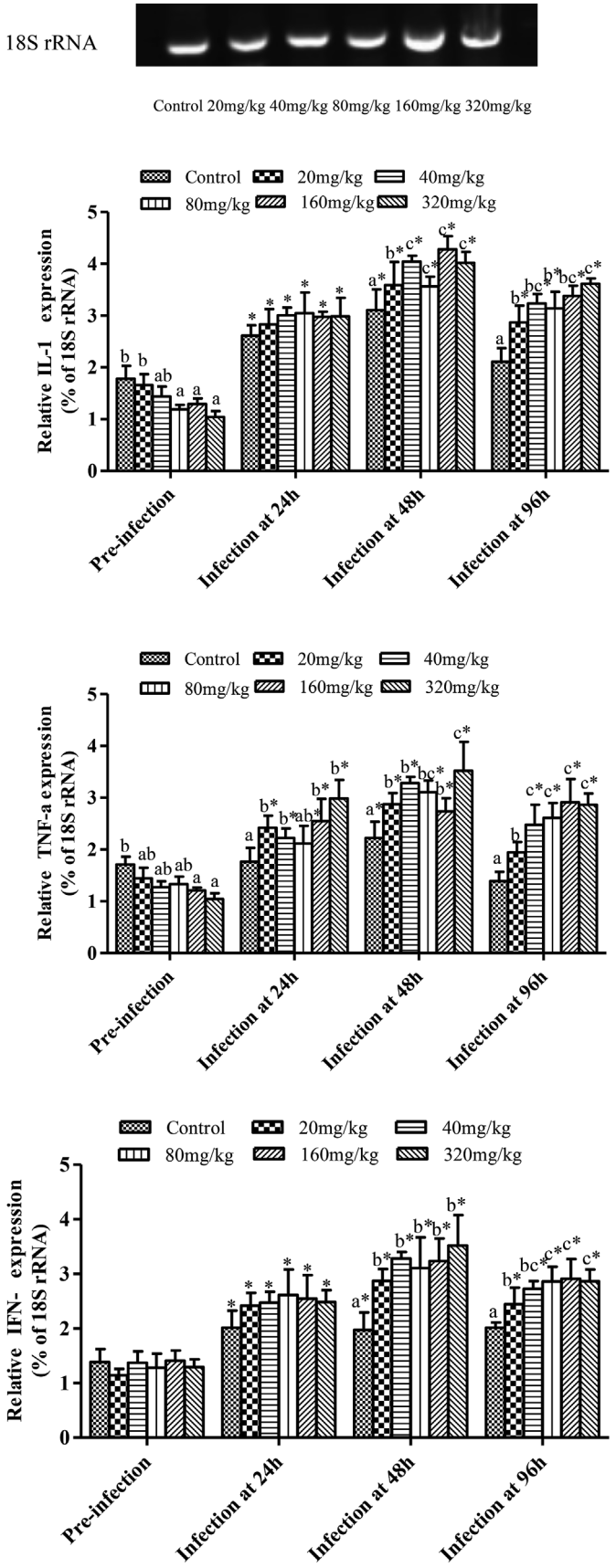

Fig. 5. Effects of dietary vitamin E levels on the inflammatory response in head kidney of genetically improved farmed tilapia fingerlings (*Oreochromis niloticus*) pre- and post- *Streptococcus iniae* infection. IL-1β: Interleukin 1β; TNF-α: tumor necrosis factor α; IFN-γ: Interferon γ.

serum. In fish, glucose levels vary greatly depending on their physiological status. Vitamin E-deficient *Piaractus mesopotamicus* showed substantial glycemic changes, whereas those fed diets containing 100 or 450 mg/kg vitamin E did not [44]. Tatina et al. [45] found that different levels of http://www.scialert.net/asci/result.php?searchin=Keywords&cat=&ascicat=ALL&Submit=Search&keyword=vitamin+C (vitamin C)" > vitamins C and E had no significant effect on plasma glucose contents. The elevated serum glucose content detected in the high-dose vitamin E-supplemented groups in our study may be related to the metabolic burden on the fish body, and the requirement for vitamin E may be limited to the fingerling stage. Andrade et al. [43] also reported that plasma glucose levels in sterlet (*Acipenser ruthenus*) were elevated in fish fed diets with high levels of vitamin E (800 and 1200 mg/kg). Changes in serum TG and TC levels can reflect the level of fat metabolism in fish. Liu et al. [46] and Chang et al. [47] reported that appropriate amounts of vitamin E reduced the serum TC and TG contents of turbot and Manchurian trout (*Brachymystax lenok*), and improved lipid metabolism in the blood. In this study, we found that the serum TC and TG levels were lower in the 160 mg/kg vitamin E group than in the control group. It may be that certain amounts of vitamin E increase the activity of TG and TC catabolic enzymes in the serum of aquatic animals, promote transport and excretion, and reduce the lipid content in the blood.

Both ALT and AST are important aminotransferases in the liver. In healthy animals, they are less active in the serum, but they are released into the serum when liver damage or lesions occur. Previous studies have reported that the intake of appropriate amounts of vitamin E in the diet of Wuchang Bream *Megalobrama amblycephala* [48] and grass carp juveniles [49] reduced serum ALT and AST activities. In the present study, serum ALT activity was significantly decreased in the 20–160 mg/kg vitamin E groups, and AST activity was significantly decreased in the 40–80 mg/kg groups. These findings suggested appropriate vitamin E supplementation helped to protect the liver of the fingerlings.

In aquatic animals, digestion occurs mainly in the digestive tract, so the activity of digestive enzymes can reflect the ability of an animal to digest its food. Sun [50] found that dietary supplementation with 195 mg/kg vitamin E significantly increased intestinal protease and amylase activities and reduced lipase activity in loach (*Misgurnus anguillicaudatus*). He et al. [51] found that 100 mg/kg vitamin E supplementation increased gastrointestinal protease and lipase activities of channel catfish (*Ictalurus punctatus*). Similarly, we found that appropriate vitamin E supplementation significantly stimulated trypsin and lipase activities in the intestine of GIFT fingerlings, which improved nutrient deposition, feed utilization, and growth.

One of the main aims of this study was to determine whether vitamin E supplementation in the diet could regulate lipid metabolism genes in GIFT fingerlings. The transcript levels of *LPL* are closely related to lipid metabolism, having not only a positive relationship with intramuscular fat deposition, but also affecting plasma lipid levels. The transcript levels of *H-FABP* and *L-FABP* are closely related to the development and function of adipose tissue. These genes are considered to be candidate genes for lipid metabolism, which affects fat deposition. We found significantly reduced transcript levels of hepatic *LPL, H-FABP,* and *L-FABP* in GIFT fingerlings in the control group, compared with the vitamin E treatment groups. There was a significant positive correlation between the transcript levels of these genes and vitamin E supplementation levels, suggesting that vitamin E affects the digestion, utilization, and transport of body tissue fat by affecting the expression of *LPL, H-FABP*, and *L-FABP* in the liver, thereby affecting serum fat deposition and HSI. However, the specific mechanism remains to be studied. Another important regulatory factor in fat metabolism is I-FABP, which is used as a marker for intestinal maturation and injury. Polymorphisms in *I-FABP* are associated with metabolic diseases such as human hyperlipidemia, type 2 diabetes, and obesity [52,53]. However, we found no significant differences in the transcript level of *I-FABP* in the liver among the treatment groups. The expression of *I-FABP* may be tissue specific, because the target organs are concentrated mainly in the intestine, so it may be less affected in the liver.

Vitamin E is the major and most effective fat-soluble antioxidant found in human RBCs. It reduces the ability of monocytes to release reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anions, thereby alleviating lipid oxidative damage in cell membranes [54]. SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen to be scavenged by CAT and GSH-Px, which decompose hydrogen peroxide to water and oxygen, thereby protecting against oxidative damage [55]. In the present study, SOD, CAT, and GSH-Px activities were significantly enhanced in the groups fed dietary vitamin E at doses from 40 to 160 mg/kg. This is a clear demonstration that dietary vitamin E can boost the antioxidant capacity of GIFT fingerlings, thereby providing protection against oxidative stress. Similar results were obtained in previous studies on black sea bream, *Sparus macrocephalus* [56] and yellow catfish [57]. Reduced lipid peroxidation may be related to high concentrations of vitamin E. It has been demonstrated that vitamin E supplementation can affect vitamin E concentrations in fish tissues. In previous studies, high vitamin E supplementation in the diet increased vitamin E concentrations in the muscle, plasma, and liver of hybrid tilapia (*O. niloticus* × *O. aureus*) [58] and rainbow trout [6]. In the present study, the α-tocopherol content was higher in the 80–320 mg/kg treatment groups than in the control group, suggesting that α-tocopherol accumulation was promoted by vitamin E supplementation. This helped to increase the antioxidative stress response and decrease lipid peroxidation (reduced MDA levels) in the liver. After infection with *S. iniae*, high concentrations of vitamin E in the tissues may help to further stimulate the antioxidant response of fingerlings. At 96 h post-*S. iniae* infection, there were higher activities of SOD, CAT, and GSH-Px in the high vitamin E-supplemented groups than in the control group. High activities of these enzymes can alleviate the oxidative damage caused by stress, reduce the accumulation of MDA, and increase the survival under stress to some extent.

In humans, vitamin E therapy, especially high-dose vitamin E therapy, has been shown to reduce the amounts of pro-inflammatory cytokines such as IL-1b, IL-6 and TNF-α in patients with cardiovascular disease [59]. Vitamin E inhibits the release of IL-1b by inhibiting the 5-lipoxygenase pathway. In mouse, vitamin E also acts as an antioxidant, inhibiting IL-1β and TNF-α and oxidative stress in murine AIDS, thereby enhancing NF-κB activation [60]. High doses of vitamin E in the diet can significantly reduce the inflammation joint markers (IL-1β) and histological expression in dogs with osteoarthritis, and can improve pain symptoms [61]. In fish, IL-1β, TNF-α, and IFN-γ represent the link between innate and acquired immunity, and play an important role in infection and autoimmunity. Until now, there have been no reports on the effect of vitamin E on pro-inflammatory factors in fish. In this study, we found that vitamin E supplementation significantly reduced the transcript levels of *IL-1β* and *TNF-α* in the head kidney of GIFT fingerlings. The increased vitamin E content in the fish tissues may have helped to inhibit the secretion of pro-inflammatory factors, thereby reducing damage caused by the inflammation response, and promoting the normal response of the immune system. Post-*S.iniae* infection, the fish in the vitamin E-treated groups showed more positive response of pro-inflammatory factors and an increased inflammatory response, for example, by increased *IL-1β, TNF-α,* and *IFN-γ* transcript levels after stress, which stimulated immune function and improved survival [62,63].

5. Conclusions

Dietary vitamin E deficiency significantly impaired the growth performance of GIFT fingerlings. Dietary vitamin E deficiency inhibited the transcript levels of *LPL, H-FABP,* and *L-FABP* in GIFT fingerlings, which affected the regulation of fat metabolism and increases serum TP

and fat deposition. Dietary vitamin E deficiency also led to increased lipid peroxidation damage in GIFT fingerlings, and inhibited the antioxidant capacity and inflammatory response post-*S.iniae* infection. Therefore, we propose that appropriate dietary vitamin E supplementation (160–320 mg/kg) at the culture stage of GIFT fingerlings will increase vitamin E accumulation in fish tissues and help to activate the immune regulatory response to bacterial infection. In further research, we will determine whether the fat content and contents of other vitamins in the diet alter these effects of vitamin E.

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Author contributions

XP and QJ conceived and designed the experiment; AW cultured the GIFT fingerlings; TYF and BJW made the feed. HJ and TYF collected samples, extracted RNA, and conducted the qRT-PCR experiments. CDJ and ZHJ measured the biochemical parameters. AW and TYF analyzed the data. QJ and AW wrote the paper with contributions from all other authors. All authors read and approved the final version of the manuscript.

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