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Dietary Protein Modifies Hepatic Glycolipid Metabolism, Intestinal Immune Response, and Resistance to *Streptococcus agalactiae* of Genetically Improved Farmed Tilapia (GIFT: *Oreochromis niloticus*) Exposed to High Temperature

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Abstract: The present study investigates the effects of dietary protein levels on glucolipid metabolism, immune function, and resistance to *Streptococcus agalactiae* of genetically improved farmed tilapia (GIFT) exposed to high temperature. Six practical diets were prepared to feed 360 fish (initial weight 43.78 ± 0.12 g) with graded protein levels (26.45%, 29.28%, 31.69%, 33.68%, 36.18%, and 38.75% dry matter). The results showed that 26.45% dietary protein significantly improved glycolysis by increasing PK mRNA levels, while the 29.28% and 31.69% dietary protein levels promoted gluconeogenesis by increasing PEPCK and G6Pase mRNA levels. For lipid metabolism, 26.45% dietary protein enhanced lipid synthesis by increasing PPAR-γ, SREBP1c, and FAS mRNA levels, while 31.69% dietary protein enhanced the level of lipolysis by increasing the PPAR-α and CPT1 mRNA levels. The highest plasma TG and TC contents were observed in the 29.28% and 31.69% dietary protein groups, respectively. In terms of antioxidants and immunity, the 31.69% dietary protein level activated the expression levels of HSP90 mRNA, thus increasing the expression levels of antioxidant-related genes (CAT, SOD, and GPx), and upregulating the anti-inflammatory factor IL-10 mRNA levels. In addition, regarding the antioxidant enzymes, the highest GSH content was found in the 29.28% dietary protein group, while the 31.69% dietary protein group had the maximum GSH-Px activity. The lowest plasma ALT and AST activities were observed in the 31.69% dietary protein group. Ultimately, the survival rate of juvenile GIFT fed 31.69% dietary protein was highest after a *Streptococcus agalactiae* challenge. Overall, 29.28–31.69% dietary protein was recommended in the diet of GIFT in a high-temperature environment.

Keywords: dietary protein; glycolipid metabolism; immune response; *Streptococcus agalactiae*; high temperature

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1. Introduction

As one of the three major nutrients, protein is the main component of the organism, and affects the metabolic and immune processes of fish. According to previous research on grass carp (*Ctenopharyngodon idellus*) [1] and top-mouth culter (*Erythroculter ilishaeformis*) [2], optimum dietary protein levels could effectively regulate glycolipid metabolism, including decreasing lipid synthesis and enhancing glycolytic capacity. Furthermore, previous studies on Japanese sea bass (*Lateolabrax japonicus*) [3] and leopard coral grouper (*Plectropomus leopardus*) [4] have shown that the appropriate dietary protein levels have a significant enhancing effect on immunity. Conversely, low-protein feeds with high levels of carbohydrates can lead to the accumulation of body fat in animals [5,6], and deficient dietary protein significantly reduces antioxidant enzyme activities and resistance to bacterial infection [7]. Moreover, excessive dietary protein levels will cause immunosuppression to some extent in fish [8,9], and excess protein will be broken down for energy consumption; then,

ammonia nitrogen and oxidation products are produced in the process, which can be toxic to the body [10].

In aquaculture, high-temperature stress will cause a stress response, resulting in a series of changes, including changes in mRNA and metabolites [11–13], which directly affect biological functions, resulting in metabolic disorders and reduced disease resistance in fish. A previous study showed that the stress response of cells under stressful conditions is closely related to alterations in energy metabolism (glucose and lipid metabolism), with high temperatures significantly altering lipid and carbohydrate metabolism [14]. Furthermore, Zhao et al. [15] reported that when turbot (*Scophthalmus maximus*) is exposed to high temperatures, lipid metabolism has an important regulatory role in stress resistance. In addition, it was also found in grass carp that high-temperature stress could decrease fatty acid synthesis and weaken carbohydrate metabolism [16]. In addition, high temperature also affects the immune system of fish, reducing nonspecific and specific immunity and leading to a decrease in resistance to pathogenic bacteria [17]. Cheng et al. [18] showed that the immunity of spotted grouper (*Epinephelus coioides*) was suppressed during heat stress and that sustained high temperatures would result in a much lower immunity against *Vibrio alginolyticus* than that at optimal temperatures. Wang et al. [19] reported that the antioxidant system of scallops (*Chlamys farreri*) is challenged by high-temperature stress and is unable to fully repair the oxidative damage caused by the stress of high temperatures combined with a bacterial infection. Thus, understanding the changes in the metabolic and immune mechanisms of the body under a high temperature environment can help reduce the potential negative effects of heat stress.

Tilapia, *Oreochromis niloticus*, is the most exported farmed fish in China, with a total production of 1.65 million tons of tilapia farmed in 2020 [20]. Tilapia was once known for its ease of culture and strong disease resistance, but in recent years, with the expansion of culture scale and the increase in culture density, diseases occur frequently and have become increasingly serious, especially *Streptococcus agalactiae* disease, which seriously threatens the healthy development of the tilapia farming industry. The optimal water temperature for tilapia growth is 29–31 °C [21]. In our previous study, it was found that the protein requirement of tilapia at high temperatures was lower than those at suitable temperatures in terms of growth performance [22]. When the water temperature is higher than 32 °C, tilapia are more likely to be infected with *Streptococcus agalactiae* [23]. As Kayansamruaj et al. [24] showed, inflammation-related genes were significantly upregulated at high temperatures, causing massive inflammatory responses and acute fish mortality. This result indicated that the resistance of tilapia to the pathogenic bacteria, *Streptococcus agalactiae*, is reduced under high temperatures, for example, due to suppression of the immune system.

To date, no studies have reported the effects of protein levels in high temperature environments on the glycolipid metabolism and immune system of tilapia, as well as the effects on antimicrobial capacity. Thus, in this study, genetically improved farmed tilapia (GIFT), one of the tilapia strains, was chosen for this experiment. Our purpose was to examine the mechanisms of dietary protein levels on the glycolipid metabolism and immune capacity under high temperature in tilapia, to improve the antimicrobial capacity of tilapia under high-temperature stress through nutritional strategies.

2. Materials and Methods

2.1. Experimental Diets

Diets with six different protein levels (26.45%, 29.28%, 31.69%, 33.68%, 36.18%, and 38.75% dry matter) were designed (Table 1). The main protein sources were the fish meal, soybean meal, rapeseed meal, cottonseed meal, and wheat flour. The lipid source is fish oil. All raw materials were first crushed and sieved through 60 mesh and then mixed with water and oil. Then, the mixture was pelletized into 2 mm-diameter pellets through a pelletizer (F-26 [II], South China University of Technology, China), air-dried at room temperature, and maintained at −20 °C until further use.

Table 1. Formulation and proximate composition (% dry matter) of experimental diets.

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fish meal [a]	2.00	2.00	2.00	2.00	2.00	2.00
Rapeseed meal [a]	25.00	25.00	25.00	25.00	25.00	25.00
Soybean meal [a]	2.00	10.00	18.00	26.00	34.00	43.00
Cottonseed meal [a]	9.00	9.00	9.00	9.00	9.00	9.00
Wheat flour [a]	35.00	29.30	23.60	17.90	12.20	6.00
Soybean oil	2.50	2.50	2.50	2.50	2.50	2.50
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin C (35%)	0.05	0.05	0.05	0.05	0.05	0.05
Vitamins premix [b]	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix [c]	2.00	2.00	2.00	2.00	2.00	2.00
Calcium dihydrogen phosphate	2.50	2.50	2.50	2.50	2.50	2.50
Rice bran	10.00	8.00	6.50	5.00	3.50	2.00
Microcrystalline cellulose	4.62	4.35	3.71	3.08	2.26	0.96
Ethoxy quinoline	0.01	0.01	0.01	0.01	0.01	0.01
Bentonite	2.00	2.00	2.00	2.00	2.00	2.00
Lysine [d]	0.32	0.26	0.14	0.00	0.00	0.00
Methionine [d]	0.33	0.38	0.40	0.42	0.47	0.48
Threonine [d]	0.17	0.15	0.09	0.04	0.02	0.00
Analyzed proximate composition						
Dry matter (%)	94.16	93.29	93.29	93.82	92.79	92.21
Crude protein (%)	26.45	29.28	31.69	33.68	36.18	38.75
Crude lipid (%)	4.56	4.35	4.65	4.39	4.60	4.31
Crude ash (%)	10.56	10.54	10.94	11.22	11.25	11.74
Crude fiber (%)	6.17	6.32	6.57	6.81	7.05	7.36
NFE [e]	46.42	42.80	39.44	37.72	33.71	30.05
Gross energy (KJ/g)	17.96	18.03	18.00	18.03	18.06	18.14

Note: The feed formulation referred to our previous study [22]. [a] Fish meal, crude protein 65.8%, crude lipid 9.5%; Rapeseed meal, crude protein 41.3%, crude lipid 6.1%; Soybean meal, crude protein 50.8%, crude lipid 4.3%; Cottonseed meal, crude protein 53.7%, crude lipid 1.4%; Wheat flour, crude protein 13.1%, crude lipid 4.0%. They were obtained from Wuxi Tongwei feedstuffs Co., Ltd., Wuxi, China. [b] Vitamins premix were obtained from HANOVE Animal Health Products Co. LTD (IU, mg/kg of diet): Vitamin A, 900,000 IU; Vitamin D, 250,000 IU; Vitamin E, 4500 mg; Vitamin K3, 220 mg; Vitamin B1, 320 mg; Vitamin B2, 1090 mg; Vitamin B5, 2000 mg; Vitamin B6, 5000 mg; Vitamin B12, 116 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg; Biotin, 50 mg Niacin acid, 2500 mg. [c] Mineral premix was obtained from HANOVE Animal Health Products Co. LTD (g/kg of diet): calcium biphosphate, 20 g; sodium chloride, 2.6 g; potassium chloride, 5 g; magnesium sulphate, 2 g; ferrous sulphate, 0.9 g; zinc sulphate, 0.06 g; cupric sulphate, 0.02 g; manganese sulphate, 0.03 g; sodium selenate, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004 g. [d] Lysine, methionine and threonine, obtained from Feeer Co., LTD (Shanghai, China). [e] NFE (nitrogen free extract, %) = dry matter (%)—(crude protein (%) + crude lipid (%) + crude ash (%) + crude fiber (%)).

The chemical compositions of the dried experimental diets were assessed based on the established methods of the AOAC [25]. The protein content was determined by the Kjeldahl method (Auto Kjeldahl apparatus: Hanon K1100 (Jinan Hanon Instruments Co., Ltd., Jinan, China)). The lipid content was determined by the Soxhlet method (Auto fat analyzer: Hanon SOX606 (Jinan Hanon Instruments Co., Ltd., Jinan, China)). The ash content was determined by the combustion method (Muffle: XL-2A (Hangzhou Zhuochi Instrument Co., Ltd., Hangzhou, China)). The fiber content was determined by the FiberCap method (Fiber analysis system (FiberCap™ 2021, FOSS, USA)). The gross energy was determined by the combustion method (Oxygen bomb calorimeter: IKA C6000 (IKA WORKS GUANGZHOU, Guangzhou, China)).

2.2. Experimental Fish and Feeding Management

Juvenile GIFT were obtained from the breeding farm of the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences. A total of 360 healthy GIFT

(average initial weight 43.78 ± 0.12 g) were evenly distributed in 18 floating cages. The floating cages with a square shape (1 m × 1 m × 1 m) were hung on the floating frame, with the buoyancy of the floating frame adjusted to allow the cages to remain in the upper layer of water. The netting of the cages is made of mesh sewn together, and the mesh area of the netting is 1 cm×1 cm, which can ensure good water exchange and prevent fish from escaping. Fish were fed to satiation three times daily for four weeks. The natural water temperature ranged from 32 °C to 36 °C, and the water quality parameters during the trial were as follows: dissolved oxygen > 6.0 mg/L, and pH was kept at 7.5–8.0.

2.3. Sampling Procedure

After 4 weeks, three fish in the cage were selected randomly to take blood samples, intestine tissues, and liver tissues. Blood samples were obtained from the caudal vein and then immediately centrifuged at 3000 rpm for 10 min at 4 °C. The separated plasma samples were stored at −20 °C until they were analyzed, and the tissues were frozen in a −80 °C freezer for later analysis.

2.4. Plasma Biochemical Analysis

Plasma biochemical parameters (TG: triglyceride, TC: total cholesterol, GLU: glucose, ALT: alanine transaminase, and AST: aspartic transaminase) were measured by a BS-400 automatic biochemical analyzer (Mindray, Shenzhen, China) using the corresponding Mindray Kits.

2.5. Analysis of Intestinal Antioxidant Indices

Intestinal antioxidant indices, including the activities of intestinal total catalase (CAT), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), and the levels of glutathione (GSH) and malondialdehyde (MDA), were assayed by relevant assay kits (Jian Cheng Bioengineering Institute, Nanjing, China).

2.6. Total RNA Extraction and Real-Time RT–PCR Analysis

First, total RNA was extracted, and the quality and quantity of the RNA were evaluated with a spectrophotometer. Finally, the reaction system was set up and run on a real-time PCR machine. The reagents and machine models used in the above process were the same as those used in our previous study [16]. Moreover, β-actin was used as the internal reference gene, and the specific primers for the target genes used are shown in Table 2. The mRNA expression levels were determined according to Pfaffl's mathematical model [26].

2.7. Streptococcus agalactiae Challenge Test

After sampling, 10 fish (average body weight 109.73 g) from each cage were challenged with *Streptococcus agalactiae* (*S. agalactiae*) in indoor recirculating culture barrels (180 L), the water temperature was controlled at 33–35 °C, the pH value ranged from 7.6 ± 0.2, and dissolved oxygen levels were maintained at 6–7 mg/L. Before the challenge experiment, the pre-experiment was carried out to determine the half-lethal concentration (1×10^6 CFU/mL) of *S. agalactiae* using a bacterial turbidimeter (SGZ-6AXJ, Yue Feng Instrument Co. Ltd., Shanghai, China). The specific method is described in our previous study [27]. Then, the fish were challenged by intraperitoneal injection with 1 mL/100 g (1% of body weight). The mortality rate within 144 h was recorded.

2.8. Data Analysis

The data were subjected to normality and homogeneity tests. The experimental data (means ± SEM) were analyzed using SPSS 24.0 statistical software for one-way analysis of variance (ANOVA). When the difference was significant ($p < 0.05$), Duncan's multiple comparisons were performed.

Table 2. Primer sequence for qRT-PCR.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
CAT [a]	GGAAGAGGATGACGAAGAG	GTTACGGCGAGATGATGT
CPT1 [b]	TCAACACCACACGCATTCCT	AAAGTAGCGCCCTTTGTGGT
FAS [c]	TCATCCAGCAGTTCACTGGCATT	TGATTAGGTCCACGGCCACA
G6Pase [d]	AGCGCGAGCCTGAAGAAGTACT	ATGGTCCACAGCAGGTCCACAT
GK [e]	GACATGAGGACATTGACAAGGGAA	CTTGATGGCGTCTCTGAGTAAACC
GPx [f]	CCAAGAGAACTGCAAGAACGA	CAGGACACGTCATTCCTACAC
HO-1 [g]	CTTGCCCGTGTGGAATCACT	AGATCACCGAGGTAGCGAGT
HSP90 [h]	ATCATCAATGTCCAGCATCA	CATCTTCGCAGCATACCA
IFN-γ [i]	ATGGCTACCACAGTGAGGGCAG	AACTCTGGGGCGACCTTTAGC
IL-8 [j]	CTGTGAAGGCATGGGTGTGGAG	TCGCAGTGGGAGTTGGGAAGAA
IL-10 [k]	CTGCTAGATCAGTCCGTCGAA	GCAGAACCGTGTCCAGGTAA
PEPCK [l]	CTGCGCAAGTACAGCAACTG	TCATGGCTTTGTCCCACTCC
PK [m]	GCACTCCTCAGCTGGTTAAT	GCAAGCACTAGAGCAGGATTT
PPAR-α [n]	TCCAAAAGAAGAACCGAAACA	TTCCACCTCTTTCTCAACCAT
PPAR-γ [o]	TTTACCCATCAAACTGACCAC	GAGGAAATGGAGGCGTAGT
SOD [p]	ACAGAAGAGAAGTATCAGGAG	CACCGTAACAGCAGACAT
SREBP1c [q]	TGCAGCAGAGAGACTGTATCCGA	ACTGCCCTGAATGTGTTCAGACA
TNF-α [r]	AAGCCAAGGCAGCCATCCAT	TTGACCATTCCTCCACTCCAGA
β-actin	CCACACAGTGCCCATCTACGA	CCACGCTCTGTCAGGATCTTCA

[a] CAT, catalase. [b] CPT1, carnitine palmitoyl transterase-1. [c] FAS, fatty acid synthase. [d] G6Pase, glucose-6-phosphatase. [e] GK, glucokinase. [f] GPx, glutathione peroxidase. [g] HO-1, heme oxygenase-1. [h] HSP90, heat shock protein 90. [i] IFN-γ, interferon γ. [j] IL-8, interleukin 8. [k] IL-10, interleukin 10. [l] PEPCK, phosphoenolpyruvate carboxykinase. [m] PK, pyruvate kinase. [n] PPAR-α, peroxisome proliferators-activated receptor-α. [o] PPAR-γ, peroxisome proliferators-activated receptor-γ. [p] SOD, superoxide dismutase. [q] SREBP1c, sterol-regulatory element binding protein 1c. [r] TNF-α, tumor necrosis factor α.

3. Results

3.1. Plasma Biochemical Composition

Table 3 presents the plasma biochemical variables. Among the plasma variables, the plasma glucose (GLU) contents were insignificant across the treatments ($p > 0.05$). The plasma triglyceride (TG) content showed an increasing tendency with increasing dietary protein levels up to 29.28% ($p < 0.05$), and decreased thereafter. The highest plasma total cholesterol (TC) content was observed in the 31.69% dietary protein group ($p < 0.05$). The group with 31.69% dietary protein in the feed had significantly decreased plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) activities ($p < 0.05$).

Table 3. Plasma biochemical parameters.

Dietary Protein (%)	GLU (mmol/L)	TG (mmol/L)	TC (mmol/L)	ALT (U/L)	AST (U/L)
26.45	16.63 ± 1.59	42.32 ± 0.86 [b,c]	3.24 ± 0.08 [a,b]	22.16 ± 2.35 [a]	118.04 ± 11.00 [a,b]
29.28	18.61 ± 0.79	45.64 ± 1.86 [c]	3.22 ± 0.07 [a,b]	25.60 ± 1.89 [a,b]	117.57 ± 13.99 [a,b]
31.69	15.35 ± 0.89	45.39 ± 2.90 [c]	3.47 ± 0.08 [b]	21.25 ± 3.68 [a]	75.60 ± 16.75 [a]
33.68	17.98 ± 1.14	39.60 ± 2.37 [a,b]	3.15 ± 0.13 [a]	22.72 ± 4.05 [a]	93.88 ± 14.74 [a,b]
36.18	18.57 ± 0.63	34.61 ± 1.18 [a]	3.15 ± 0.06 [a]	36.59 ± 3.32 [b,c]	132.18 ± 18.08 [b,c]
38.75	16.35 ± 1.12	35.44 ± 1.44 [a]	3.16 ± 0.08 [a]	46.10 ± 5.75 [c]	170.29 ± 20.01 [c]

Data are expressed as means with SEM (n = 9). Means in the same column with different superscripts [a,b,c] are significantly different ($p < 0.05$).

3.2. Intestinal Enzyme and Antioxidant Status

Table 4 shows that the GSH contents and GSH-Px activities were affected by dietary protein levels ($p < 0.05$). The highest GSH content was observed in the 29.28% dietary protein group, while the 31.69% dietary protein level yielded the largest GSH-Px activity. In addition, the other intestinal antioxidant enzyme activity indices (CAT, T-SOD, and MDA) were not affected ($p > 0.05$).

Table 4. Antioxidant enzyme activities of GIFT fed with diets containing six levels of dietary protein under high temperature.

Dietary Protein (%)	CAT (U/mg Protein)	T-SOD (U/mg Protein)	MDA (nmol/mg Protein)	GSH (μmol/g Protein)	GSH-Px (U/mg Protein)
26.45	1.68 ± 0.14	0.83 ± 0.06	0.25 ± 0.04	21.64 ± 1.64 ^{a,b}	6.59 ± 0.67 ^{b}
29.28	1.61 ± 0.12	0.73 ± 0.05	0.26 ± 0.06	24.99 ± 1.37 ^{b}	6.70 ± 0.61 ^{b}
31.69	1.60 ± 0.08	0.74 ± 0.06	0.21 ± 0.04	22.64 ± 2.29 ^{a,b}	7.42 ± 0.77 ^{b}
33.68	1.51 ± 0.14	0.70 ± 0.03	0.16 ± 0.02	18.27 ± 1.71 ^{a}	6.17 ± 0.64 ^{a,b}
36.18	1.46 ± 0.09	0.71 ± 0.03	0.31 ± 0.03	17.71 ± 1.33 ^{a}	5.56 ± 0.53 ^{a,b}
38.75	1.53 ± 0.09	0.68 ± 0.03	0.27 ± 0.05	17.27 ± 1.76 ^{a}	4.52 ± 0.44 ^{a}

Data are expressed as means with SEM (n = 9). Means in the same column with different superscripts ^{a,b} are significantly different ($p < 0.05$).

3.3. Gene Expression Analysis of Glucose Metabolism

Figure 1 shows the results of the gene expression analysis of the glucose metabolism-related genes. The mRNA expression levels of PK were significantly upregulated by 26.25% dietary protein ($p < 0.05$, Figure 1B). The GK mRNA showed the same phenomenon as PK, but the difference was not significant ($p > 0.05$, Figure 1A). The highest PEPCK and G6Pase mRNA levels were observed in the 29.28% and 31.69% dietary protein groups, respectively ($p < 0.05$, Figure 1C,D).

3.4. Gene Expression Analysis of Lipid Metabolism

Figure 2 shows the results of the gene expression analysis of the lipid metabolism-related genes. Dietary protein (31.69%) significantly upregulated PPAR-α mRNA expression levels ($p < 0.05$, Figure 2A), and CPT1 mRNA showed the same phenomenon ($p < 0.05$, Figure 2B). The PPAR-γ, SREBP1c and FAS mRNA expression levels decreased with increasing dietary protein levels, and the 26.45% dietary protein group produced the peak values ($p < 0.05$, Figure 2C–E).

3.5. Gene Expression Analysis of HSP90 and Antioxidant Status

Figure 3 shows the results of the gene expression analysis of HSP90 and antioxidant-related genes. The HSP90 mRNA expression levels increased with increasing dietary protein levels up to 31.69% ($p < 0.05$, Figure 3A) and decreased thereafter.

The expression levels of the antioxidant-related genes CAT, SOD, and GPx increased with increasing dietary protein levels up to 31.69%, and then decreased. The maximum levels were found in the 31.69% dietary protein group ($p < 0.05$, Figure 3C–E). However, HO-1 mRNA was not affected by the protein treatments ($p > 0.05$, Figure 3B).

3.6. Gene Expression Analysis of Immunity

Figure 4 shows the results of the gene expression analysis of immunity-related genes. Dietary protein levels did not markedly affect the TNF-α, IFN-γ, and IL-8 mRNA expression levels ($p > 0.05$, Figure 4A–C). In addition, the mRNA expression level of IL-10 reached a maximum value of 31.69% dietary protein ($p < 0.05$, Figure 4D).

3.7. Streptococcus agalactiae Challenge Test

Figure 5 shows the survival rate of the GIFT fed with different dietary protein levels with the *Streptococcus agalactiae* challenge after 144 h. The highest survival rate of GIFT was observed among fish given food with 31.69% dietary protein at 144 h ($p < 0.05$).

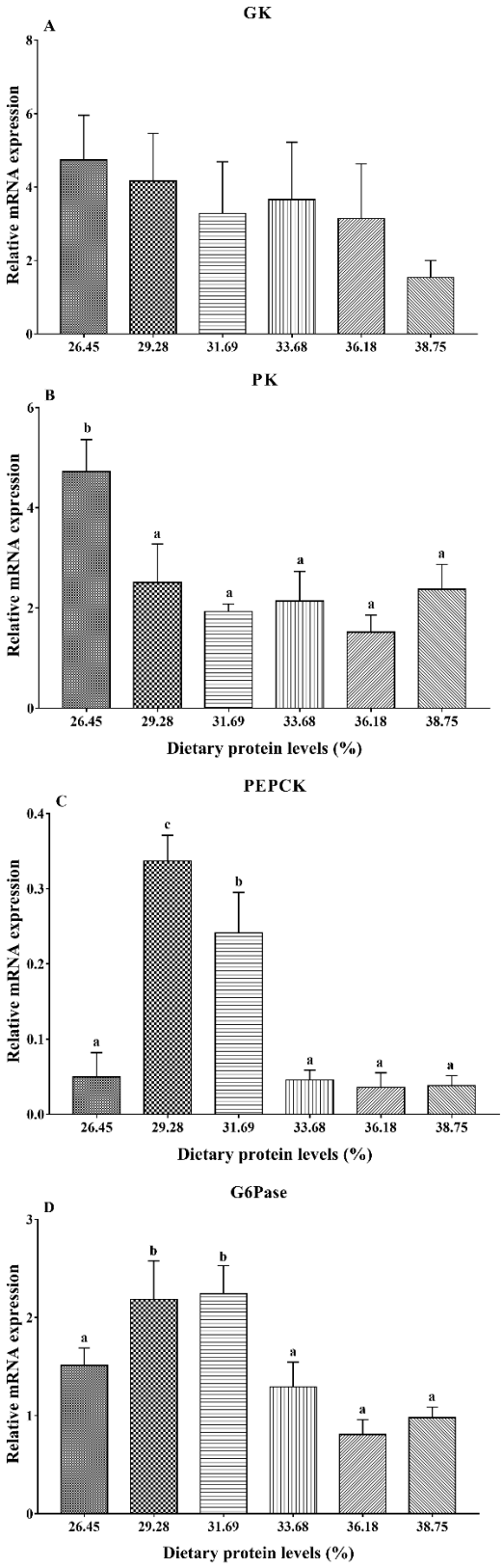

Figure 1. Relative mRNA expressions of glucose metabolism-related genes with dietary protein levels. (**A**) GK; (**B**) PK; (**C**) PEPCK; (**D**) G6Pase. Data are expressed as means with SEM (n = 9). Values with different superscripts are significantly different ($p < 0.05$).

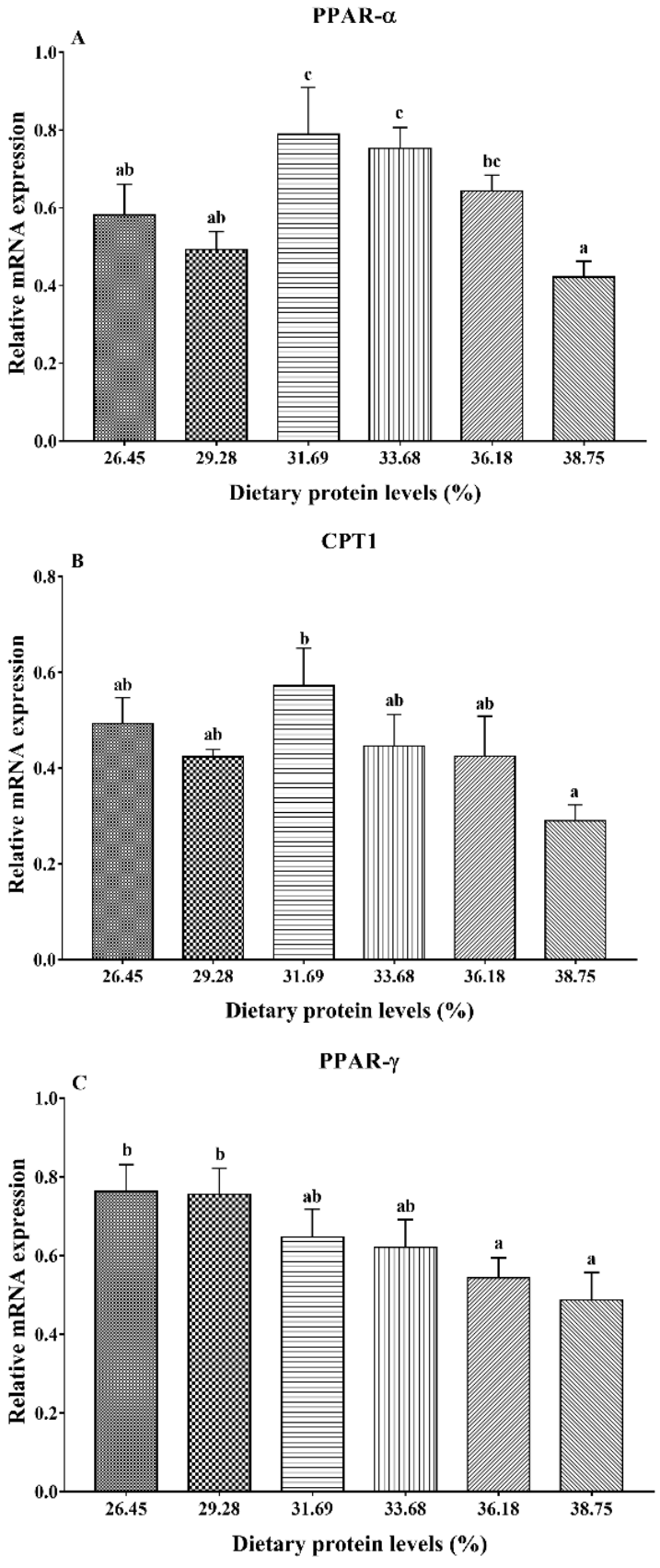

Figure 2. *Cont.*

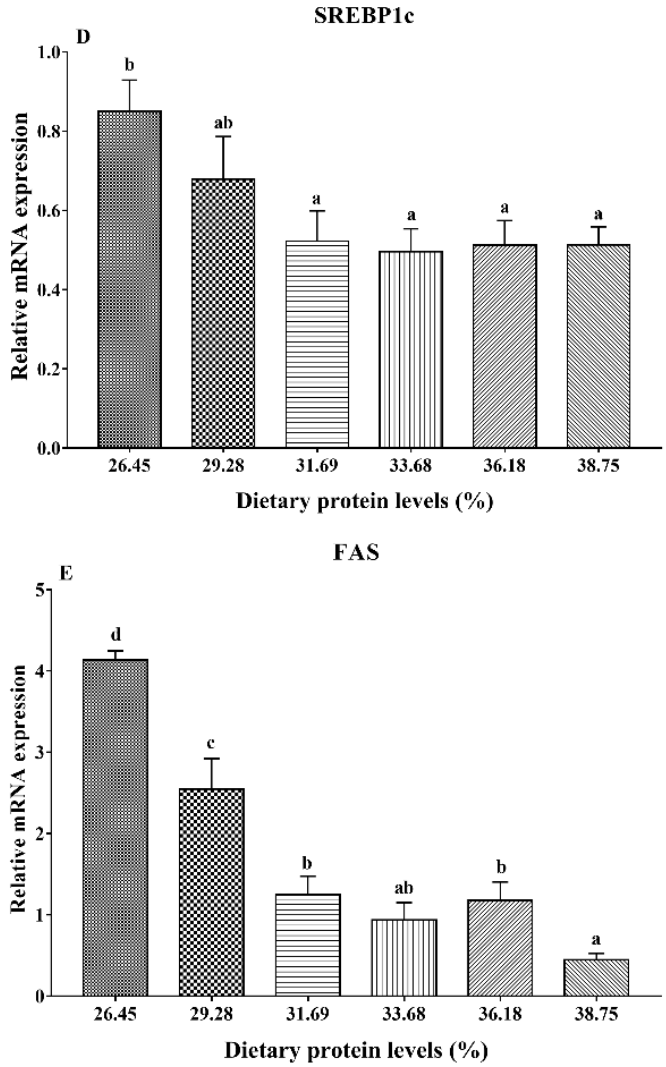

Figure 2. Relative mRNA expressions of lipid metabolism-related genes with dietary protein levels. (**A**) PPAR-α; (**B**) CPT1; (**C**) PPAR-γ; (**D**) SREBP1c; (**E**) FAS. Data are expressed as means with SEM (n = 9). Values with different superscripts are significantly different ($p < 0.05$).

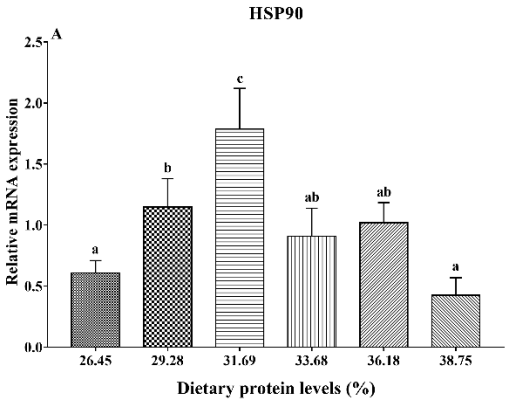

Figure 3. *Cont.*

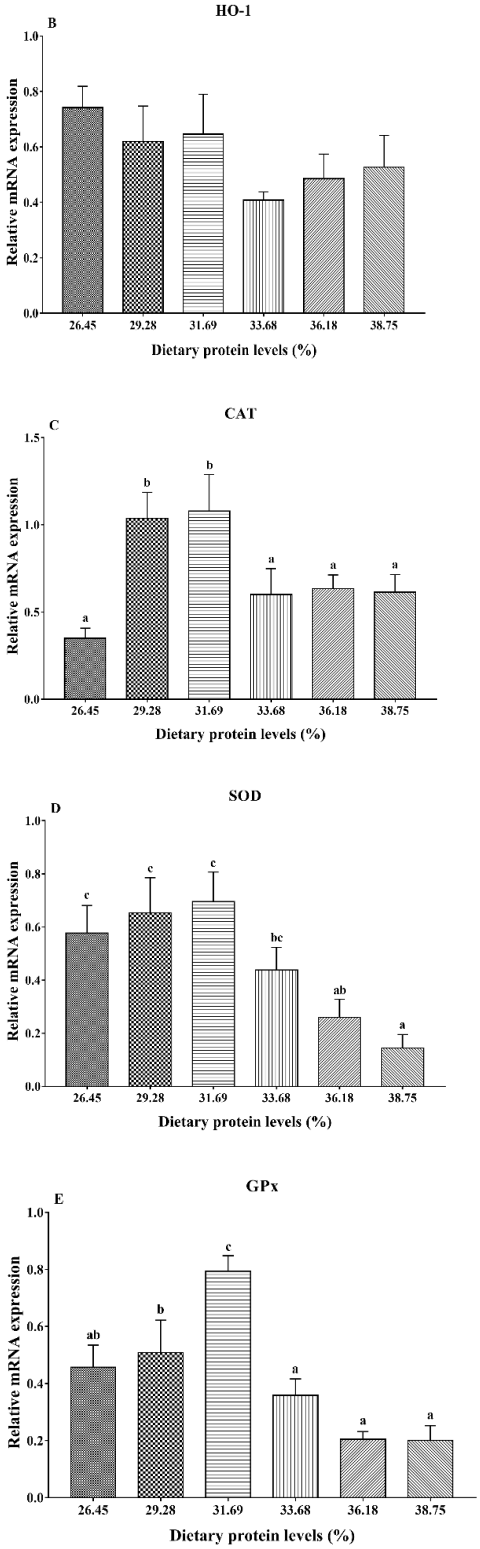

Figure 3. Relative mRNA expressions of HSP90 and antioxidant-related genes with dietary protein levels. (**A**) HSP90; (**B**) HO-1; (**C**) CAT; (**D**) SOD; (**E**) GPx. Data are expressed as means with SEM (n = 9). Values with different superscripts are significantly different ($p < 0.05$).

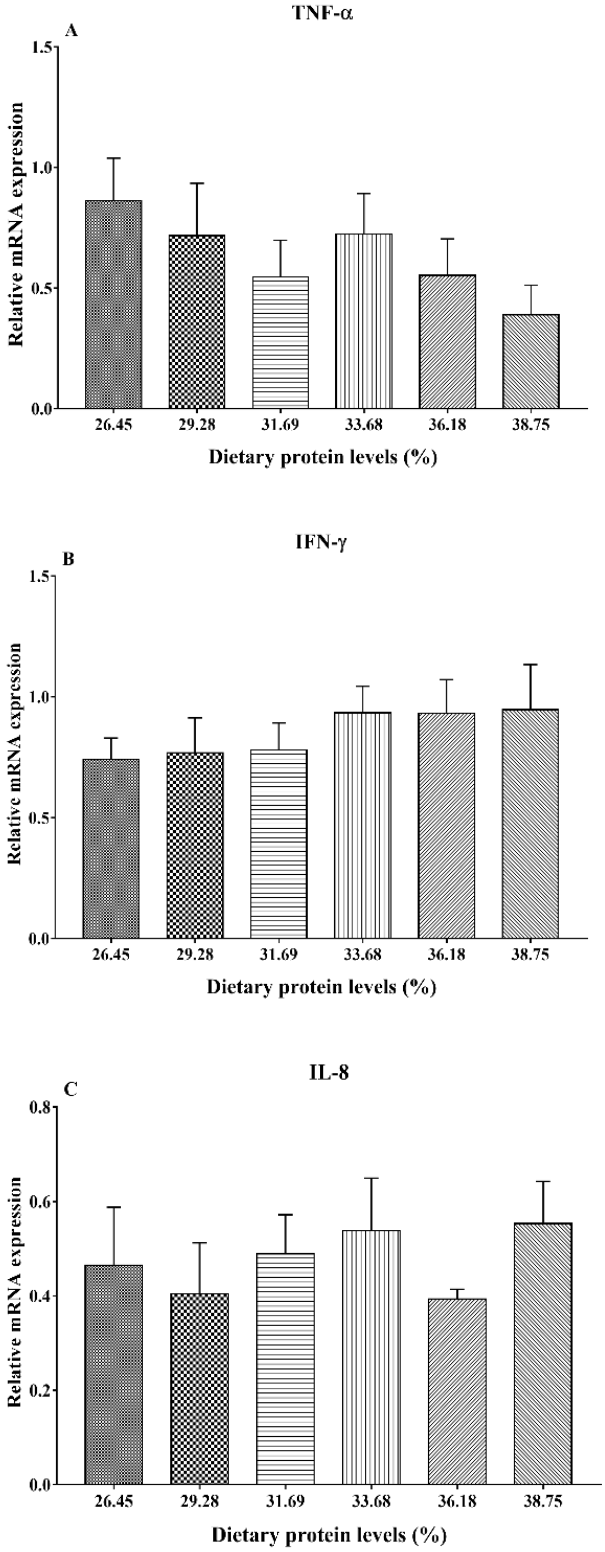

Figure 4. *Cont.*

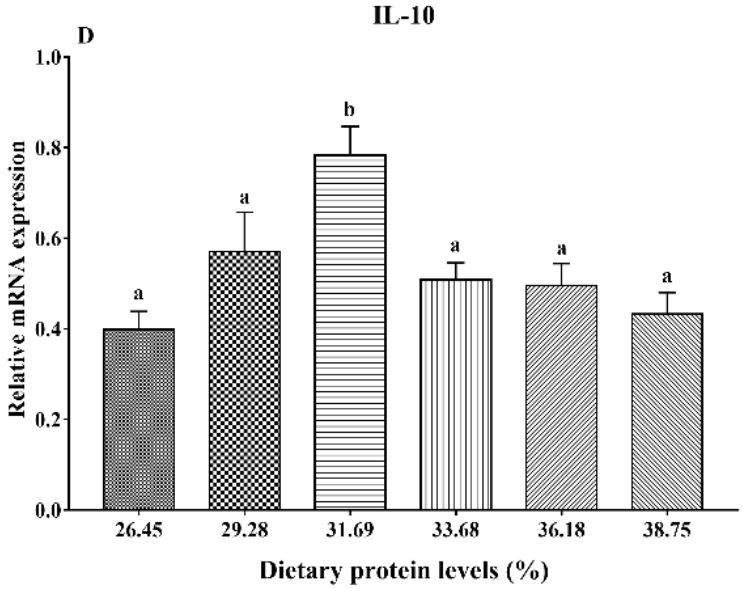

Figure 4. Relative mRNA expressions of antioxidant and immune-related genes with dietary protein levels. (**A**) TNF-α; (**B**) IFN-γ; (**C**) IL-8; (**D**) IL-10. Data are expressed as means with SEM (n = 9). Values with different superscripts are significantly different ($p < 0.05$).

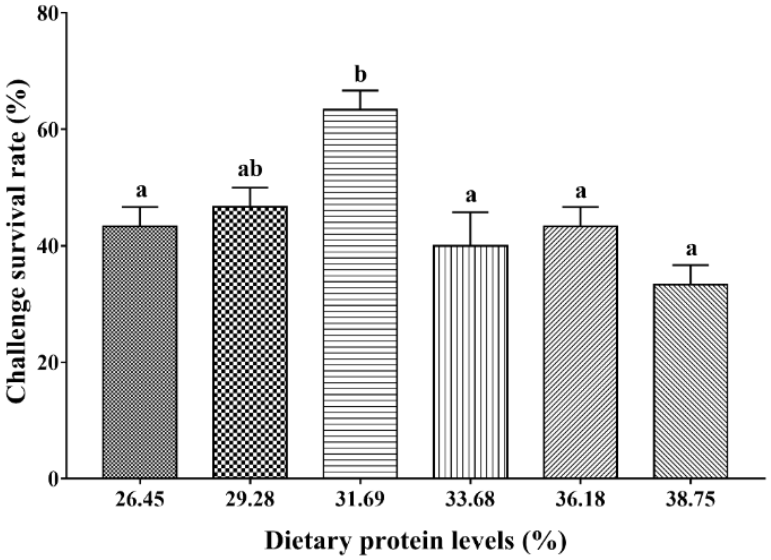

Figure 5. The survival rate of GIFTs fed with different dietary protein levels with *Streptococcus agalactiae* challenge after 144h. Data are expressed as means with SEM (n = 3). Values with different superscripts are significantly different ($p < 0.05$).

4. Discussion

This study demonstrated that dietary protein had significant effects on the glucose and lipid metabolism of GIFT under high temperatures. In this study, low-protein diets increased the expression of glycolysis-related genes, and 26.45% dietary protein resulted in the highest PK mRNA expression levels. It was found that increasing the carbohydrate levels in the feed induces an increase in glycolytic enzyme activities in the liver, which was consistent with a previous study on rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) [28]. In addition, the gluconeogenesis-related genes PEPCK and G6Pase were activated in the 29.28% and 31.69% dietary protein groups, respectively, which were higher than the levels in the 26.45% dietary protein group. This finding indicated

that metabolic regulation occurred in GIFT when fed low-protein diets (26.45%, 29.28%, and 31.69%) with high carbohydrate levels, which means that low-protein diets enable rapid adaptation of hepatic glucose metabolism and increased enzyme activity under high temperatures, thus increasing the availability of glucose [29]. Cai et al. [30] reported that a low-protein diet may be more appropriate at higher temperatures due to alterations in liver metabolism. Plasma GLU is an important energy supplier in the fish body and can directly provide energy for various life activities of the fish [31]. In our current study, the plasma GLU contents did not differ significantly among all groups, which was different from other reports that blood glucose levels tend to increase with carbohydrate contents [32,33]. Our current study suggested that dietary protein levels did not influence the energy homeostasis of juvenile GIFT, which may be caused by external temperature factors. However, studies investigating the effect of dietary protein on the plasma parameters of fish under high-temperature stress are still limited, and further investigation is needed.

Regarding lipid metabolism, the PPAR signaling pathway plays an important role in regulating the transcription of genes [34]. PPAR-α and PPAR-γ regulate lipid catabolism and synthesis in lipid metabolism homeostasis, respectively [35]. In this experiment, the expression levels of PPAR-γ mRNA presented a decreasing trend with increasing dietary protein levels. In addition, the downstream factor FAS also showed the same tendency: the highest mRNA level was found in the lowest protein diet (26.45%), which was higher than that in the other groups. This result was in line with our previous study [1] on grass carp, which indicated that low-protein diets could cause an accumulation of lipids in the liver. Furthermore, SREBP1c has a positive correlation with lipid synthesis-related genes [36], which also presented the same phenomenon as PPAR-γ. In addition, the lipolysis-related gene PPAR-α showed a trend of increasing and then decreasing, and the 31.69% dietary protein group achieved the peak value. As a downstream signaling molecule, CPT1 also regulates fatty acid β-oxidation [37], and it exhibited the same trend in response to dietary protein treatments. Our current study indicated that appropriate dietary protein (31.69%) could promote lipolysis and release more energy. As the main sources of blood lipids, the plasma TG and TC contents in the low-protein diets (26.45%, 29.28%, and 31.69%) were higher than those in the high-protein diets (33.68%, 36.18%, and 38.75%), and the highest levels were observed in the 29.28% and 31.69% dietary protein groups. The reason for this result may be the high carbohydrate contents in the low-protein diets, and, thus, the fish can synthesize fat from carbohydrates [38].

High-temperature stress also suppresses the immune system of the fish body while facilitating the growth and reproduction of pathogenic bacteria and reducing the resistance of tilapia to pathogenic bacteria [39]. In the current study, dietary protein levels also had significant effects on the antioxidant status and immune response of GIFT under high temperatures. Under stressful conditions, HSP90 acts as a regulatory enzyme to prevent irreversible protein aggregation and to improve cellular tolerance to stress [40]. Our experimental results showed that an appropriate dietary protein level (31.69%) could activate the expression of HSP90, which was higher than the levels with other dietary treatments. This result indicated that an appropriate dietary protein level (31.69%) enhances the ability to scavenge free radicals and improves the immunity of GIFT under high-temperature stress. Rokutan et al. [41] revealed that increased oxygen radicals can act as a stressor to induce the production of HSP, while HSP can increase the level of peroxidase, inhibit the key enzymes that produce oxygen radicals, and ultimately scavenge oxygen radicals. In addition, HSP significantly attenuates protein exudation during inflammation and inhibits the inflammatory response [42].

High-temperature stress can cause oxidative stress in fish, leading to oxidative damage. The body responds by regulating gene expression levels and regulating the key antioxidant enzymes for oxidative stress [43]. According to previous studies, high expression of antioxidant enzymes can prevent oxidative stress in fish [44]. Numerous studies have shown that the activity of antioxidant enzymes decreases under high-temperature stress, indicating that fish are unable to eliminate the damage produced by peroxides [45,46].

Interestingly, appropriate dietary protein levels could improve the antioxidant enzymes in some fish species [47,48], which supports our findings. In the current study, the highest GSH content was found in the 29.28% dietary protein group, while the 31.69% dietary protein group yielded the maximum GSH-Px activity. In addition, the corresponding antioxidant enzyme genes (CAT, SOD, and GPx) increased continuously with increasing dietary protein levels; however, they started to decrease when the dietary protein level exceeded 31.69%. As downstream regulators of HSPs [49], CAT, SOD, and GPx mRNA showed the same tendency as HSP90, which suggested that the 31.69% dietary protein treatment could significantly improve the intestinal antioxidant capacity of GIFT.

On the other hand, high levels of HSP90 produce a strong stimulus to the body and enhanced immune function [50]. According to previous studies, heat shock proteins reduce damage to the body from inflammatory responses by inhibiting reactive oxygen species and cytokines [42,51]. In this experiment, the pro-inflammatory factors TNF-α, IFN-γ, and IL-8 were not affected by dietary protein treatments under high temperature, while the anti-inflammatory factor IL-10 showed a similar tendency to HSP90, and the highest expression level was found in the 31.69% dietary protein group. Li et al. [52] reported that simultaneous activation of HSPs and anti-inflammatory factors helped to improve the immunity of common carp (*Cyprinus carpio* L.), which was consistent with our findings in tilapia. In addition, plasma ALT and AST activities can be used as indicators of fish health [53]. In this study, the 31.69% dietary protein group had the lowest ALT and AST activities, while the highest levels were observed with the highest protein diet (38.75%), which suggested that high-protein diets are not good for fish health under high-temperature stress.

In addition, tilapia are more susceptible to *Streptococcus agalactiae* infection in high-temperature environments. Thus, it is important to select the appropriate dietary protein level to improve the antibacterial ability. In this study, the survival rate of juvenile GIFT fed 31.69% dietary protein was significantly higher than that of other groups (except 29.28% dietary protein) after a *Streptococcus agalactiae* challenge, which suggested that 31.69% dietary protein level could also enhance the immunity and antioxidant capacity to resist pathogenic bacterial infection.

A previous report has shown that suitable water temperatures for tilapia growth range from 29 to 31 °C [21]. Under appropriate temperature, numerous studies on tilapia have shown that the optimal protein levels of tilapia range from 33% to 35% [54–56], which is higher than the optimal protein requirement (29.28–31.69%) under high temperature in this study. The differences could be explained by high temperatures beyond the optimal growth temperatures inducing retarded growth; meanwhile, high-protein feeds are more detrimental to protein utilization [57]. Combined with the immunization results of this experiment, it can be concluded that adequate reduction of dietary protein levels at high temperatures is more beneficial for tilapia to improve its disease resistance than under suitable temperature conditions.

5. Conclusions

Under a high-temperature environment, low-protein feed (26.45%) enhanced the level of glycolysis and lipid synthesis, and supplementation with appropriate protein (29.28% and 31.69%) enhanced the level of gluconeogenesis and lipolysis. In addition, appropriate dietary protein (29.28% and 31.69%) can effectively improve the antioxidant capacity, enhance immune function, and strengthen the antibacterial capacity of GIFT at high temperature (Figure 6). Overall, 29.28–31.69% dietary protein was recommended in the diet of GIFT in a high-temperature environment.

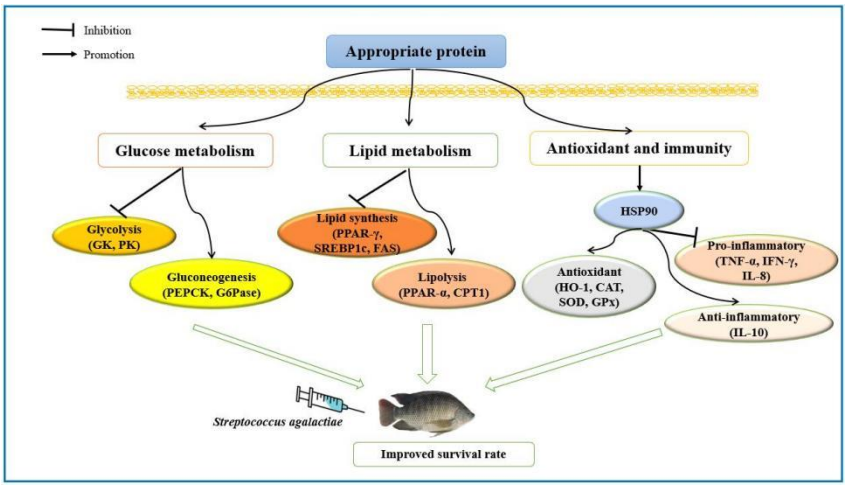

Figure 6. Regulation mechanism of improving health status by appropriate dietary protein levels in GIFT under high-temperature environment.

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Institutional Review Board Statement: The study was conducted according to Standardization Administration of China protocols and guidelines (GB/T 35892-2018), and approved by the Institutional Animal Care and Ethics Committee of Nanjing Agricultural University (Permit number: SYXK (Su) 2011–0036).

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the manuscript, tables and figures.

Conflicts of Interest: The authors declare no conflict of interest.

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