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A deficiency or an excess of dietary threonine level affects weight gain, enzyme activity, immune response and immune-related gene expression in juvenile blunt snout bream (*Megalobrama amblycephala*)

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ABSTRACT

A feeding trial was conducted to investigate the impacts of deficient and excess dietary threonine levels on weight gain, plasma enzymes activities, immune responses and expressions of immune-related genes in the intestine of juvenile blunt snout bream. Triplicate groups of fish (initial weight 3.01 ± 0.01 g, 30 fish per tank) were fed with deficient (0.58%), optimum (1.58%) and excess (2.58%) threonine level diets to near satiation four times a day for 9 weeks. A mixture of L-amino acids was supplemented to simulate the whole body amino acid pattern of blunt snout bream, except for threonine. The results showed that both deficiency and excess threonine level diets significantly ($P < 0.05$) reduced the weight gain of blunt snout bream. Excess dietary threonine level triggered plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities ($P < 0.05$); whereas superoxide dismutase (SOD) activity was not significantly influenced by imbalanced-dietary threonine level ($P > 0.05$). Plasma complement component 3 (C3) and component 4 (C4) concentrations were significantly depressed by the deficiency of dietary threonine ($P < 0.05$). Dietary threonine regulated the target of rapamycin (TOR), eukaryotic translation initiation factor 4E-binding protein 2 (4E-BP2), tumour necrosis factor alpha (TNF-α) and copper–zinc superoxide dismutase (Cu/Zn-SOD) gene expressions in the intestine of blunt snout bream, which may go further to explain the adverse effects of a deficient and/or an excess dietary threonine level on growth, immunity and health of fish. Furthermore, the present study also suggests that an optimum dietary threonine could play an important role in improving growth, enhancing immune function and maintaining health of fish.

1. Introduction

Amino acids have a central role in the defense mechanisms since they are involved in the synthesis of an array of proteins such as antibodies and in the control of key immune regulatory pathways. Recently, the role of dietary amino acids on fish immune response has hardly received attention. Some essential amino acids are important regulators of key metabolism pathways that are necessary for maintenance, growth, reproduction, and immunity in organisms, thus maximizing efficiency of food utilization, enhancing protein accretion, reducing adiposity and improving health [1]. Amino acid imbalances as well as their antagonisms also could affect nutrient utilization and can have a direct consequence on immune organs and responses [2]. Threonine is the third limiting amino acid for growing fish fed with low protein diets and involved in many physiological and biochemical processes, including in growth and immune functions [2–6].

The gastrointestinal tract (GIT) is an organ with multiple functions in nutrition as well as immunity. The organization of the gut

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associated lymphoid system of teleost intestine is not as complex as that in mammals, but has a more diffused pattern [7]. The gut mucosa is rich in immune cells such as lymphocytes, plasma cells, eosinophilic (mast cell-like) granulocytes, and macrophages and can elicit local responses [8]. The GIT mucosal surface is a natural interface where the intestinal microbiota and antigen cross-talk with the host fish [9]. The microbes, either commensal or pathogenic, are in direct contact with the gut mucosa and the gut associated lymphoid tissue distinguishes between them to initiate either tolerance or immune response. Among essential amino acids, threonine is extracted in greater proportion by the small intestine in mammals, suggesting that threonine is involved in intestinal functionality and maintenance [10,11]. Additionally, intestinal mucins are particularly enriched in threonine (up to 30% of the amino acid composition) [12]. Thus, threonine may play an important role in the immune functions of fish, which has not yet been studied.

Many parameters including antioxidants and antibody response have been used as good immunological indicators in fish. The changes in enzymatic activity of aquatic organisms have been widely used to demonstrate tissue damage and diagnoses of fish diseases [13,14]. Fish antioxidant capacity includes enzymatic and non-enzymatic antioxidant defenses [15]. Some cytokine and immune-related genes have been identified and characterized in fish [16–19]. In mammals, the mammalian mTOR pathway has emerged as a key regulator of the inflammatory response in monocytes, macrophages and peripheral myeloid dendritic cells [20]. Wacyk et al. [21] implied that dietary branched-chain amino acid (BCAA) increases hepatic TOR gene expression in rainbow trout. To our knowledge, there is no information reported regarding the effect of threonine on the TOR signaling pathway as inflammatory response in intestine of fish. In addition, inflammatory cytokines play an important role in immunity of fish [16]. The expression of TNF-α mRNA in mouse liver was reduced by BCAA supplementation [22].

Blunt snout bream is a principal herbivorous finfish in Chinese freshwater polyculture systems as a delicacy. During the last decade production of this species has expanded rapidly and its yield reached approximately 0.7 million tons in 2012 in China [23]. However, in recent years, disease outbreaks showed an increasing trend in cultured blunt snout bream and resulted into high mortality, especially during summer [24]. Nevertheless, few nutritional studies have been reported about this species. To our knowledge, there are no reports in literature regarding the effects of dietary threonine on immune responses and expressions of some immune-related genes in this fish. Based on our unpublished results the dietary threonine requirement of juvenile blunt snout bream was estimated to be 1.57% of the diet, corresponding to 4.62% of the dietary protein (Habte-Tsion et al., unpublished data). We hypothesized that an imbalanced-threonine in a diet may influence weight gain, immune responses and expressions of immune-related genes in fish. Indeed, this study was carried out to investigate the impacts of a deficient and/or an excess dietary threonine level on the weight gain, enzymes activities, immune responses and expressions of immune-related genes in the intestine of juvenile blunt snout bream.

2. Materials and methods

2.1. Experimental Diet

Three isonitrogenous and isoenergetic (34% crude protein and 17 kJ g^{-1} energy) experimental diets were formulated to contain deficient (0.58%), optimum (1.58%) and excess (2.58%) levels of dietary threonine (Table 1). The diets were supplemented with 0, 1.0, and 2.0% L-threonine of the diet. Diets were made isonitrogenous and isoenergetic by adjusting the level of glutamic acid. L-amino acid mixtures were prepared taking into account the amount of amino acids contributed by fish meal, casein and gelatin. A mixture of crystalline L-amino acids was supplemented to simulate the whole body amino acid pattern of blunt snout bream, except for threonine (Table 2). All the ingredients were ground into powder and thoroughly mixed with soybean oil and water and then pelleted (1.5 mm and 2.0 mm in diameter) using Lab extruder (Science and Technology Industrial Factory of South China University of Technology, China). Pellets were air-dried to approximately 10% moisture, sealed in plastic bags and stored at −15 °C until used.

2.2. Experimental facility and fish husbandry

Fish husbandry was conducted in an indoor freshwater recirculating system consisting of 9 fiberglass tanks (300-L each) with equal supplemental aeration and water flow (3 L min^{-1}). Juvenile of blunt snout bream were obtained from Nan Quan Station—Freshwater Fisheries Research Center (FFRC) and acclimatized with the experimental facilities and condition for two weeks. At the start of the trial, similar size of fish (initial weight, 3.01 ± 0.01 g) were selected and restocked at a stocking density of 30 fish per tank. Three tanks were randomly arranged and assigned to each diet. The fish were fed with the respective diet to near satiation four

Table 1
Ingredients and composition of the optimum diet.

Ingredients (%)		Proximate composition (%, DM)[a]	
Fish meal[b]	5.00	Crude protein (%)	34.42
Casein[c]	12.00	Crude lipid (%)	7.32
Gelatin[d]	3.00	Gross Energy (KJ g^{-1})	16.96
Soya-bean oil[e]	6.00	Threonine (%)	1.58
Soyabean lecithin[e]	1.00		
Amino acid premix[f]	15.29		
Choline chloride[e]	0.25		
Vitamin C[g]	0.15		
Vitamin and Mineral Premix[h]	2.00		
Monocalcium phosphate[i]	2.75		
Dextrin[i]	35.00		
Microcrystalline cellulose[i]	8.49		
Carboxy methyl cellulose[i]	6.32		
Ethoxyquin[i]	0.50		
Glutamic acid	2.25		

[a] Values for the proximate composition of the test diets are means of triplicate analyses.
[b] Crude protein 67.4%, crude lipid 9.3%, provided by Tongwei Feed Group Co., Ltd., Jiangsu, PR China (origin Copeinca, Lima, Peru).
[c] Crude protein 90.2%, purchased from Huaan Biological Products Co., Ltd., Gansu, PR China.
[d] Crude protein 91.3%, purchased from Shanghai Zhan Yun Chemical Co., Ltd. PR China.
[e] Provided by Cargill, Shanghai, PR China.
[f] Amino acid premix (g/100 g diet): L-histidine, 0.31; L-isoleucine, 0.68; leucine, 0.87; L-lysine, 1.09; L-methionine, 0.43; L-phenylalanine, 0.66; L-threonine, 0.71; L-valine, 0.56; L-aspartic acid, 1.46; serine, 0.55; glycine, 1.37; alanine; 1.25; L-cystine 0.14; L-tyrosine, 0.27; tryptophan, 0.12; glumatic acid, 1.11; proline 0.12. Amino acids obtained from Feeer Co., Ltd., Shanghai, PR China.
[g] 35% ascorbic acid equivalent, provided by Tongwei Feed Group Co. Ltd., Jiangsu, PR China.
[h] Vitamin(IU or mg/kg of premix) and mineral premixes (g/kg of premix): Vitamin A , 900000 IU ; Vitamin D , 250000 IU ; Vitamin C 10000 mg; Vitamin E , 4500 mg ; Vitamin K3 , 220 mg ; Vitamin B1 , 320 mg ; Vitamin B2 , 1090 mg ; Vitamin B6 , 5000 mg ; Vitamin B12 , 116 mg ; biotin , 50 mg ; Pantothenate , 1000 mg ; Folic acid , 165 mg ; Choline , 60,000 mg ; Inositol , 15000 mg ; Niacin acid , 2500 mg; CuSO4•5H2O , 2.5g ; FeSO4•7H2O , 28g ; ZnSO4•7H2O , 22g ; MnSO4•4H2O , 9g ; Na2SeO3 , 0.045g ; KI , 0.026g ; CoCl2•6H2O , 0.1 g. Provided by Wuxi Hanove Animal Health Products Co. Ltd., Jiangsu, PR China.
[i] Provided by Guangzhou Hinter Biotechnology Co., Ltd., Guangdong, PR China.

Table 2
Amino acid composition of ingredients (g 100 g^{-1}dry matter).

Amino acid	Amount in				Total	34% WBP[e]
	12 g C[a]	3 g G[b]	5 g FM[c]	CAAP[d]		
IAA[f]						
Arginine	0.34	0.19	0.18	1.30	2.01	2.01
Histidine	0.27	0.01	0.11	0.38	0.76	0.76
Isoleucine	0.50	0.04	0.14	0.81	1.49	1.49
Leucine	0.97	0.07	0.23	1.13	2.40	2.40
Lysine	0.80	0.09	0.23	1.32	2.43	2.43
Methionine	0.29	0.02	0.09	0.50	0.90	0.90
Phenylalanine	0.50	0.05	0.14	0.79	1.47	1.47
Threonine	0.44	0.04	0.11	0.00	0.58	1.41
Valine	0.62	0.06	0.16	0.73	1.57	1.57
DAA[g]						
Aspartic acid	0.77	0.12	0.27	1.68	2.84	2.84
Serine	0.56	0.07	0.12	0.70	1.45	1.45
Glycine	0.19	0.55	0.19	1.56	2.50	2.50
Alanine	0.34	0.22	0.20	1.39	2.14	2.14
Cystine	0.03	0.00	0.02	0.15	0.19	0.19
Tyrsine	0.55	0.02	0.10	0.41	1.07	1.07
Gulmatic acid	2.23	0.26	0.42	1.73	4.64	4.64
Proline	0.95	0.31	0.10	0.55	1.92	1.92

Tryptophan could not be detected after acid hydrolysis.
[a] C, casein.
[b] G, gelatin.
[c] FM, fish meal.
[d] CAAP, crystalline amino acid premix.
[e] WBP, whole body protein.
[f] IAA, indispensable amino acids.
[g] DAA, dispensable amino acids.

times a day for 9 weeks. Uneaten diet was siphoned from each tank before feeding on a daily basis. During the experimental period water quality parameters were kept as follows: temperature was constant (26 ± 1 °C), pH was 7.0–7.5, ammonia nitrogen was lower than 0.05 mg L^{-1} and dissolved oxygen was not less than 6.0 mg L^{-1}. Photoperiod was natural (light–dark cycle) throughout the experiment period. The use of experimental fish was under scientific research protocols of Chinese Academy of Fishery Sciences (CAFS) and Ministry of Agriculture, PR China that complied with all relevant local and/or international animal welfare laws, guidelines and policies [25].

2.3. Sample collection and analysis

2.3.1. Sample collection

At the end of the experiment, fish were starved for 24 h to evacuate the alimentary tract contents prior to sampling. Three fish from each tank were sampled and anaesthetised with 100 mg L^{-1} MS-222. Blood samples were obtained from the caudal vein and then centrifuged at 3000 × *g* at 4 °C for 10 min to prepare the plasma. The plasma was stored at −80 °C for subsequent plasma biochemical measurement. Meanwhile, the sampled fishes were dissected and then proximal intestine (PI), mid intestine (MI) and distal intestine (DI) samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent assay of gene expressions. At last all fish were weighed to calculate the weight gain.

2.3.2. Biochemical analysis

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined by a colorimetric test kit (Mindray Bio Medical Co., Ltd., Shenzhen, China) according to Reitman and Frankel [26]. Superoxide dismutase (SOD) activity was detected according to the method described by Zhou et al. [27], by its ability to inhibit superoxide anion generated by a xanthine and xanthine oxidase reaction system using an SOD detection kit (Nanjing Jiancheng Bioengineering Institute, China).

Complement component 3 (C3) and 4 (C4) levels were determined according to the method described by Tang et al. [28], using a reagent kit from Zhejiang Yilikang Biotech Co., Ltd., P.R. China. In brief, plasma samples were automatically mixed with an antibody afforded by a test kit and then an antigen–antibody complex was produced. The change in absorbance was read at 340 nm over a fixed-time interval, which is directly proportional to the complement C3 and C4 concentrations in the sample. Complement C3 and C4 concentrations were calculated in mg L^{-1}. All assay kits were specially designed for fish detection.

2.3.3. Real-time PCR analysis

Total RNA was extracted from proximal intestine (PI), mid intestine (MI) and distal intestine (DI) using an RNAiso plus kit (Takara, Dalian, China). The quality and quantity of RNA was assessed by spectrophotometry at 260 and 280 nm. Subsequently, complementary DNA (cDNA) was synthesized using a PrimeScript™ RT reagent kit (Takara, Dalian, China), according to the manufacturer's instructions. Briefly, oligo dT primers (50 μM) were used to reverse transcribe respective RNA in the presence of PrimeScript™ RT enzyme mix I, 5 × PrimeScript™ buffer, and RNase free distilled water at 37 °C for 15 min, following inactivation at 85 °C for 5 s. Specific primers for TOR, 4E-BP2, TNF-α and Cu/Zn-SOD genes were designed according to the partial cDNA sequences of the TOR, 4E-BP2, TNF-α and Cu/Zn-SOD genes using the *Megalobrama amblycephala* transcriptome analysis [29], and primer for β-Actin was designed using the published sequences of blunt snout bream (GeneBank accession no. AY170122.2) (Table 3). All primers were synthesized by Shanghai Biocolor, BioScience & Technology Company, China.

Real-time PCR was used to determine mRNA levels using PrimerScript™ reagent kit (Takara, Dalian, China). Real-time PCR were performed for TOR and 4E-BP2 according to standard protocols with the primers presented in Table 3. Briefly, cDNA (2.0 μl) was reacted with 10.0 μl SYBR® Premix Ex Taq II (2 ×), 0.8 μl forward primer (10 μM), 0.8 μl reverse primer (10 μM), 0.4 μl ROX reference dye or dye II (50 ×), and 6.0 μl RNase-free distilled water in a 20 μl final reaction volume. Real-time PCR was performed in a Mini Opticon Real-Time Detector (BIO-RAD, USA). The thermocycling conditions for TOR, 4E-BP2, TNF-α and Cu/Zn-SOD were the following: initiated with a denaturation step at 95 °C for 30 s, followed by forty cycles at 95 °C for 5 s, 60 °C for 34 s and 95 °C for 30s, 95 °C for 3 s, 60 °C for 30 s, respectively. The melting curve analysis was performed over a range of 50–95 °C to verify that a single PCR product was generated. The expression levels of TOR, 4E-BP2, TNF-

Table 3
Real-time PCR primer sequences.

Target gene	Primer sequence	Amplicon length (bp)
TOR		
Forward	5′-TTTACACGAGCAAGTCTACGGA-3′	22
Reverse	5′-CTTCATCTTGGCTCAGCTCTCT-3′	22
4E-BP2		
Forward	5′-ATGTCGTCCAGTCGTCAGTTT-3′	21
Reverse	5′-AGGAGTGGTGCAATAGTCGTG-3′	21
TNF-α		
Forward	5′-GTGGCCAGGGCAGAAGAAGA-3′	20
Reverse	5-CGCTCATCCACAGCCACATC-3	20
Cu/Zn-SOD		
Forward	5′-AGTTGCCATGTGCACTTTTCT-3′	21
Reverse	5′-AGGTGCTAGTCGAGTGTTAGG-3′	21
β-Actin		
Forward	5′-TCGTCCACCGCAAATGCTTCTA-3′	22
Reverse	5′-CCGTCACCTTCACCGTTCCAGT-3′	22

α and Cu/Zn-SOD genes were normalized to the expression levels of a housekeeping blunt snout bream gene, β-actin. The expression results were analyzed using the $2^{-\Delta\Delta CT}$ method after verification that the primers were amplified with an efficiency of approximately 100% [30]. PCR efficiency was determined with a standard curve using a serial dilution of cDNA: $\Delta\Delta C_T = (C_{T,Target} - C_{T,\beta\text{-actin}})_{time\ x} - (C_{T,Target} - C_{T,\beta\text{-actin}})_{time\ o}$.

2.3.4. Statistical analysis

Data were statistically analyzed using SPSS version 19 (SPSS, Chicago, IL, USA), then subjected to one-way analysis of variances (ANOVA) followed by LSD multiple comparisons. Significant differences among the group means were further compared using Duncan's multiple range tests. The effects of the deficient, optimum and excess dietary threonine levels on weight gain was estimated using second-degree polynomial regression analysis as described by Zeitoun et al. [31]. $P < 0.05$ was considered statistically significant. Results were expressed as mean ± SE.

3. Results

3.1. Effects of deficient and excess dietary threonine on weight gain

Fig. 1 shows the effects of deficient and excess dietary threonine level on weight gain of juvenile blunt snout bream fed with diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) threonine levels for 9 weeks. Based on the second-degree polynomial regression analysis, the lowest weight gain was obtained in the groups fed with deficient and excess dietary threonine levels, compared to the optimum threonine level ($P < 0.05$).

3.2. Effects of deficient and excess dietary threonine on plasma enzymes activities and immune responses

Enzymes activities and immune responses in plasma of juvenile blunt snout bream fed with diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) threonine levels for 9 weeks are presented in Table 4. The highest activities of ALT and AST were obtained in fish fed with excess dietary threonine level ($P < 0.05$). The plasma SOD activity was not significantly influenced by imbalanced-dietary threonine level ($P > 0.05$). The lowest plasma complement C3 concentration was found in fish fed with deficient dietary threonine level ($P < 0.05$). Complement C4 concentration for the group fed with deficient dietary threonine level was significantly ($P < 0.05$) lower than the groups fed with diets containing optimum and excess threonine levels.

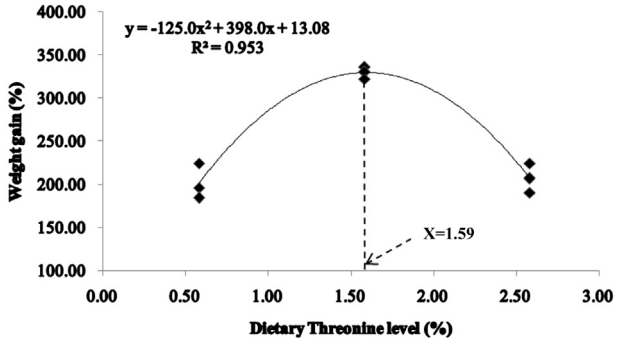

Fig. 1. Second-degree polynomial relationship of weight gain to dietary threonine levels for juvenile blunt snout bream fed with diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) threonine levels for 9 weeks; where "X" represents the dietary threonine level for the maximum weight gain of juvenile blunt snout bream.

Table 4
Enzymes activities and immune responses in plasma of juvenile blunt snout bream fed with diets containing deficient, optimum and excess threonine levels for 9 weeks[a].

Enzymes	Dietary threonine level (%)		
	0.58	1.58	2.58
ALT[b] (U L^{-1})	4.55 ± 0.60[a]	4.32 ± 0.46[a]	7.20 ± 0.61[b]
AST[c] (U L^{-1})	93.97 ± 6.12[a]	103.92 ± 8.90[a]	134.05 ± 3.11[b]
SOD[d] (U mL^{-1})	86.89 ± 2.55	87.14 ± 2.76	93.16 ± 3.15
C3[e] (mg L^{-1})	258.24 ± 7.58[a]	489.11 ± 24.91[b]	487.78 ± 25.59[b]
C4[f] (mg L^{-1})	105.39 ± 4.15[a]	123.42 ± 10.58[a,b]	131.32 ± 5.37[b]

[a] Data are mean value of three replicates ± SEM. Means in the same row with different superscripts are significantly ($P < 0.05$) different.
[b] ALT, alanine aminotransferase.
[c] AST, aspartate aminotransferase.
[d] SOD, superoxide dismutase.
[e] C3, complement component 3.
[f] C4, complement component 4.

3.3. Effects of deficient and excess dietary threonine on TOR and 4E-BP2 gene expressions

Fig. 2 shows TOR and 4E-BP2 mRNA expressions in the PI, MI and DI of juvenile blunt snout bream fed with deficient (0.58%), optimum (1.58%) and excess (2.58%) dietary threonine levels for 9 weeks. TOR mRNA level in PI was the lowest for fish fed with excess dietary threonine level ($P < 0.05$). TOR mRNA level in MI and DI was the lowest for group fed with diet containing deficient threonine level followed by excess dietary threonine level ($P < 0.05$). 4E-BP2 mRNA level in PI was the highest for fish fed with excess dietary threonine level ($P<0.05$). 4E-BP2 mRNA in MI significantly ($P<0.05$) decreased from deficient to excess dietary threonine levels, and no significant difference was found in DI among the groups ($P > 0.05$). TOR mRNA mRNA level showed an inverse trend with 4E-BP2 mRNA expression.

3.4. Effects of deficient and excess dietary threonine on TNF-α gene expression

TNF-α expression in the PI, MI and DI of juvenile blunt snout bream fed with diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) threonine levels for 9 weeks are presented in Fig. 3. TNF-α mRNA level in PI was significantly ($P < 0.05$) influenced by excess dietary threonine level. TNF-α mRNA level in MI was the highest for the groups fed with deficient and excess dietary threonine levels, compared to the fish fed with optimum dietary level ($P < 0.05$). No significant difference was found in TNF-α mRNA level of DI among the groups ($P > 0.05$).

3.5. Effects of deficient and excess dietary threonine on Cu/Zn-SOD gene expression

Fig. 4 shows Cu/Zn-SOD mRNA expression in the PI, MI and DI of juvenile blunt snout bream fed with diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) threonine levels for 9 weeks. Cu/Zn-SOD mRNA level in PI was the highest for fish fed with diet containing an excess threonine level ($P < 0.05$). No significant difference was found in Cu/Zn-SOD mRNA level of MI among the groups ($P > 0.05$). Significantly ($P < 0.05$) higher Cu/Zn-SOD mRNA level in DI was found in the groups fed with deficient and excess dietary threonine levels, compared to the control group.

4. Discussion

The present study showed that significant depression of weight gain in the group fed with deficient (0.58%) dietary threonine level,

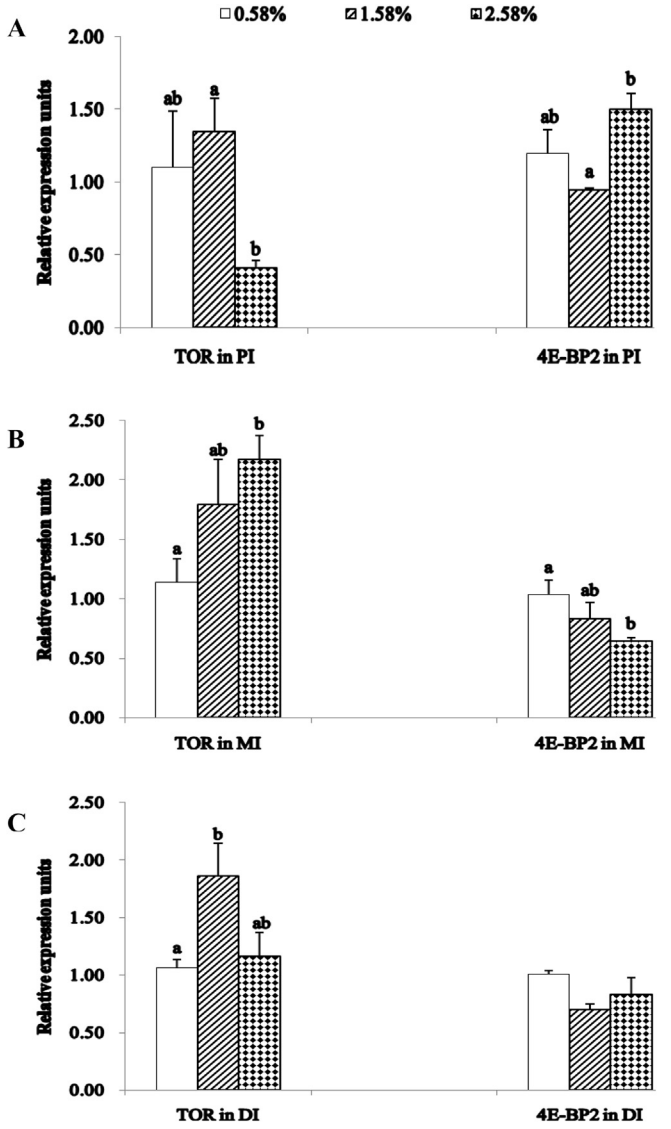

Fig. 2. Relative expression of target of rapamycin (TOR) and eukaryotic translation initiation factor 4E-binding protein 2 (4E-BP2) genes in the proximal intestine (PI) (**A**), the mid intestine (MI) (**B**), and distal intestine (DI) (**C**) of juvenile blunt snout bream fed diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) levels of threonine for 9 weeks. Values are means for nine fish per treatment, with standard errors represented by vertical bars ($n = 9$). Mean values with unlike small letters were significantly different ($P < 0.05$).

which indicates that a deficiency of dietary threonine may result a reduction in growth performance and feed utilization efficiency that cause an increase in oxidation of other essential and nonessential amino acids present at normal levels in a diet [32,33]. An anorectic status followed by weight loss also occurred in Indian major carp [5], grass carp [6], common carp [34], *Catla catla* [35], Japanese flounder [36] and Indian catfish [37] in response to test diets lacking or low in threonine but containing otherwise adequate levels of all nutrients.

In this study, the optimum (1.58%) dietary threonine level supported the highest weight gain; whereas fish fed with excess (2.58%) dietary threonine level showed remarkable reduction in weight gain. The reduction in weight gain with an excess dietary threonine level might be due to: (1) extra energy expenditure for deamination; (2) disturbance of absorption and utilization of other amino acids; (3) lower palatability of the diet; or (4) toxic effects

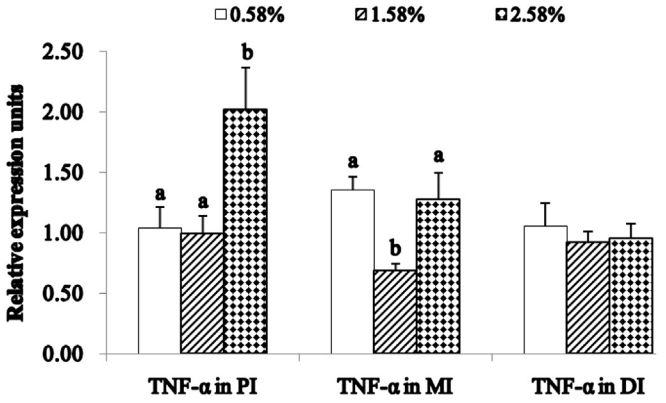

Fig. 3. Relative expression of tumour necrosis factor α (TNF-α) gene in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of juvenile blunt snout bream fed diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) levels of threonine for 9 weeks. Legends are the same as in Fig. 2.

and stress [38,39]. Adverse effects of excess dietary threonine on growth of other fish species also reported in different studies [5,35,36,40]. Additionally, in rapidly growing tissues of young pigs, either an excess and/or a deficiency of dietary threonine decreased protein synthesis [41], which elucidates a mechanism for the low growth performance of animals fed a threonine -imbalanced diet.

During amino acid metabolic processes, AST and ALT are two important enzymes that are usually present within cell membrane, cytoplasm and mitochondria. Generally, high plasma AST and ALT activities indicate that a weakening or damage of normal liver function [42,43]. Moreover, AST and ALT are known to play a key role in mobilizing L-amino acids for gluconeogenesis and functions as link between carbohydrate and protein metabolism under altered physiological condition [6]. In the present study, plasma AST and ALT activities were significantly influenced by imbalanced dietary threonine and the highest activities were obtained in fish fed with excess (2.58%) dietary threonine level. These results indicated that an excess dietary threonine level may have negative impacts on fish health. Similarly, excess dietary threonine level markedly affected AST activity in grass carp [6], and AST and ALT activities in Pacific white shrimp [44]. On the contrary, excess dietary threonine did not significantly influence the activity of ALT in grass carp [6]. These discordances could be due to phylo-genetically distinct of species and diets composition.

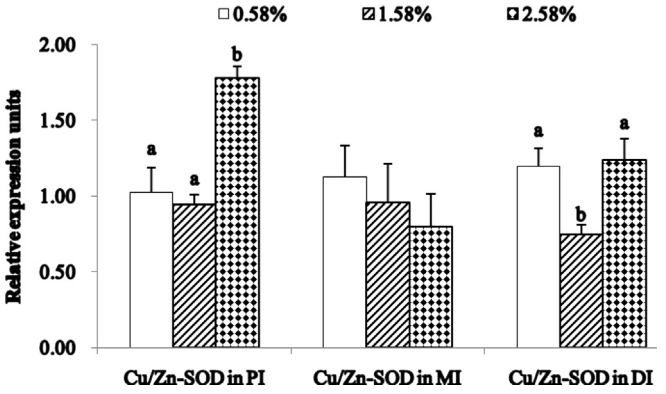

Fig. 4. Relative expression of copper–zinc superoxide dismutase (Cu/Zn-SOD) gene in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of juvenile blunt snout bream fed diets containing deficient (0.58%), optimum (1.58%) and excess (2.58%) levels of threonine for 9 weeks. Legends are the same as in Fig. 2.

SOD is one of the critical antioxidant enzymes in the body [45], which plays an important role in the self-defense and immune systems [46,47], and it is considered to be an indicator of the antioxidant status of organisms and a biomarker of oxidative stress [48,49]. In fish, antioxidant defenses can be influenced by both intrinsic (systematic position, age, feeding behavior, food consumption and diet type) and extrinsic (toxins present in the water, seasonal, and daily changes in dissolved oxygen and water temperature) factors [50–53]. The lowest activity of SOD was observed in shrimp fed with a low-threonine diet, and the activity increased when dietary threonine levels increased from 1.28% to 2.30% [44]. Nevertheless, in the present study, plasma SOD activity was not significantly influenced by the deficiency and/or excess dietary threonine levels, which indicated that the threonine-imbalance had no influence on the oxidative stress in juvenile blunt snout bream. Comparable result was reported for juvenile Nile tilapia fed with graded levels of dietary threonine [54].

A role for threonine in the immune response is consistent with the reports of increased health problems in threonine deficient animals [55]. For instance, threonine deficient mammals have been shown to be more susceptible to tumors and Plasmodium berghei infection [56,57]. Additionally, pigs fed with a diet deficient in threonine had low antibody levels to bovine serum albumin [55]. Bhargava et al. [58] suggested two mechanisms for the effect of dietary threonine on antibody production in their study with chickens; such as firstly threonine may directly influence the process of antibody production, and secondly or alternatively threonine may act on this production indirectly by modifying virus replication. Besides, it is well known that fish complement can lyse foreign cells and opsonize foreign organisms for destruction by phagocytes [59], while C3 and C4 play a pivotal role in the activation of complement system [60]. In this study, low complement C3 and C4 concentrations were obtained in the group fed with deficient dietary threonine level, which indicated that threonine deficiency has adverse effect on the immune functions of fish as it does in mammals. To our knowledge, this study is the first to determine the effect of dietary threonine on the complementary C3 and C4 in fish. However, the exact mechanism by which threonine mediates immune functions in fish is not known and needs to be investigated.

The mTOR pathway plays an important role in the inflammatory response in mammals [20] as well as in fish [61]. The TOR signaling pathway also plays an important role in regulating protein synthesis [62,63]. The 4E-BP family of translational repressors is a well-known target of mTOR kinase [64]. In this study, dietary threonine up regulated TOR mRNA expression levels in the intestine (PI, MI and DI) of blunt snout bream to some level and then reduced in the PI and DI of fish fed with excess (2.58%) threonine level, while the reverse was found in 4E-BP2 mRNA levels. Similar trends were found in intestine of Jian carp fed with diets containing graded dietary isoleucine levels [65]. Nevertheless, the specific mechanism by which dietary threonine regulates the expression of TOR and 4E-BP2 in fish remains to be studied. Our results also showed that patterns of difference in mRNA levels of 4E-BP2, the inhibitor of translation, were properly opposite to TOR mRNA levels in the PI, MI and DI of blunt snout bream, suggesting that threonine might decrease the inhibition of translation and increase TOR activity, thus improving proteins synthesis. This study investigated that threonine can regulate the TOR pathway in the intestine of fish; thus it could play an important role in the defense mechanisms since it is involved in the synthesis of an array of proteins such as antibodies and in the control of immune regulatory pathways.

Inflammation characterizes the innate immune response and is primarily mediated by cytokines [16]. In vertebrates, TNF-α is pro-inflammatory cytokines, which initiates inflammatory processes and accelerate additional inflammatory processes by inducing other inflammatory molecules [66]. In this study, the intestinal pro-inflammatory cytokine TNF-α mRNA level was the highest in fish fed with deficient (0.58%) and excess (2.58%) dietary threonine levels. Besides, the lowest TNF-α mRNA level was found in the intestine of fish fed with optimum (1.58%) dietary threonine level and showed an inverse pattern with TOR mRNA levels. Similar trends of TNF-α mRNA were reported in juvenile Jian carp fed with graded levels of dietary isoleucine [61]. The inverse relationship between TOR mRNA and TNF-α mRNA levels indicated that an important role of mTOR pathway in the inflammatory responses. Besides, our results investigated that an optimum dietary threonine level could reduce the inflammatory responses in intestine of fish, whereas a deficiency and/or an excess dietary threonine could trigger the inflammatory responses. To our knowledge, the present study is the first to investigate the influence of dietary threonine on the inflammatory responses in fish. Similarly, optimum dietary isoleucine reduced the expression TNF-α mRNA in head kidney of Jian carp [61]. Additionally, BCAA supplementation reduced TNF-α mRNA expression level in the liver of mice [22]. However, the underlying mechanism by which dietary threonine influences the cytokine expression in fish is still unknown and needs further investigation.

SODs are ubiquitous and known as three forms (based on the metal cofactor in active sites) such as copper/zinc (Cu/Zn-SOD), iron SOD (Fe-SOD) and manganese SOD (Mn-SOD) in eukaryotes. In animals, two kinds of SODs have been commonly well-studied: cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD [67]. Regarding the SOD activity along with the transcriptional regulation, Cu/Zn-SOD, the most abundant and ubiquitous isoform, which may have great physiological significance. For instance, transcription of *sod1*, the gene coding for Cu/Zn-SOD, is regulated tightly in response to diverse stimuli, including stress, pro-inflammatory cytokines, and growth factors [68,69]. In our study, Cu/Zn-SOD mRNA level was up-regulated by the excess (2.58%) dietary threonine in PI, and by both deficient (0.58%) and excess (2.58%) dietary threonine in DI of juvenile blunt snout bream, which showed a similar pattern of expression with pro-inflammatory cytokine TNF-α. The present study suggests Cu/Zn-SOD mRNA levels might be regulated in response of pro-inflammatory cytokines. However, understanding the underlying mechanism requires further study.

5. Conclusion

These results indicate that both deficiency and excess of dietary threonine reduced the weight gain of juvenile blunt snout bream. Excess dietary threonine level triggered plasma AST and ALT activity. Dietary threonine deficiency depressed plasma complement C3 and C4 concentrations. Dietary threonine regulates TOR, 4E-BP2, TNF-α and Cu/Zn-SOD gene expressions in intestine, which may go further to explain the adverse effects of threonine-imbalanced diets on growth, immunity and health of fish. Additionally, an inverse relationship was found between the expression of TOR and TNF-α, whereas a similar expression pattern was found between TNF-α and Cu/Zn-SOD. Nevertheless, the mechanisms by which dietary threonine regulates the expression of these genes and their relationship in fish needs further study. Furthermore, this study suggests that an optimum dietary threonine could play an important role in promoting growth, enhancing immune function and maintaining health of fish.

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