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Effects of dietary tryptophan levels on antioxidant status and immunity for juvenile blunt snout bream (*Megalobrama amblycephala*) involved in Nrf2 and TOR signaling pathway

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ABSTRACT

Dietary administration of tryptophan has been proved improving growth performance of fish. An 8-week feeding trial was conducted to investigate the effects of dietary tryptophan level on antioxidant capacity and immune response through Nrf2 and TOR signaling pathway. The results showed that, 0.08% tryptophan level significantly increased plasma aspartate aminotransferase (AST), while immunoglobulin M (IgM) and alkaline phosphatase (ALP) were strikingly increased by 0.40% level. The level of plasma complement component 3 (C3), alanine aminotransferase (ALT) and albumin (ALB) were independent of tryptophan supplementation. Total superoxide dismutase (T-SOD), catalase (CAT), total antioxidant capacity (T-AOC) and glutathione (GSH) activity were increased with increasing dietary tryptophan level until 0.40% and then decreased, while the level of malondialdehyde (MDA) showed a reverse trend. 0.19% and 0.28% tryptophan level significantly improved the glutathione peroxidase 1 (GPx-1) activity. Compared with 0.08% dietary tryptophan level, 0.40% level significantly improved nuclear factor erythroid 2-related factor 2 (Nrf2), GPx, manganese superoxide dismutase (Mn-SOD), CAT and transforming growth factor-β (TGF-β) mRNA level, while Kelch-like ECH-associated protein 1 (Keap1) and interleukin 1β (IL-1β) mRNA level were significantly decreased. The relative expression of copper zinc superoxide dismutase (Cu/Zn-SOD), heme oxygenase-1 (HO-1), target of rapamycin (TOR), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), protein kinase B (Akt) and interleukin 10 (IL-10) were significantly improved by 0.28% diet, while the mRNA level of tumor necrosis factor-α (TNF-α) and nuclear factor-kappa B (NF-κB) were increased by 0.08% diet. Interleukin 8 (IL-8) mRNA level was not significantly affected by dietary tryptophan. Based on MDA and T-SOD value, the optimal dietary tryptophan level of juvenile blunt snout bream was determined to be 0.33% (1.03% of dietary protein) and 0.36% (1.13% of dietary protein), respectively, using quadratic regression analysis.

1. Introduction

Tryptophan is an essential amino acid for fish [1]. As a structural component of proteins, tryptophan promotes protein synthesis and deposition, which is important for fish growth [2]. Tryptophan is also a precursor of serotonin, an important neurotransmitter, which affects feed intake and regulates aggressive behavior in fish [3]. Previous studies in fish have been reported that optimal tryptophan was beneficial for fish growth [3–6]. Furthermore, in some fish species, it was reported that tryptophan deficiency could lead to poor growth, scoliosis, lordosis, eye cataracts and a derangement of mineral metabolism [7,8], such as rainbow trout (*Salmo gairdneri*) [9], fingerling Rohu (*Labeo rohita*) [10] and fingerling Indian catfish (*Heteropneustes fossilis*) [11]. Recently, various studies on fish showed that fish nutrition, growth, immune response and antioxidant ability are closely related [12–15]. Likewise, available evidences showed that tryptophan could improve body immune response and antioxidant capacity [16,17].

The antioxidant defense is effective in protecting against injury in

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immune organs of fish [18,19]. Fish antioxidant capacity comprises antioxidant enzymes (such as superoxide dismutase (SOD), catalase (CAT)) and non-enzymic defense (such as glutathione (GSH), malondialdehyde (MDA)) [20,21]. Increased enzyme activity is resulted from increased synthesis of enzyme protein, which is related to gene transcription [22]. Fish similar to mammals, nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is the major cellular protective mechanism against oxidative stress [23], which could regulate antioxidant enzymes and corresponding genes [24,25]. Kelch-like-ECH-associated protein 1 (Keap1), the cytosolic repressor of Nrf2, blocks Nrf2 nuclear transfer and expression [26,27]. In mammals and fish, it has been reported that amino acids and their metabolism up-regulate Nrf2 mRNA level [28,29]. It was reported that a correlation exists between tryptophan and the gene expression of related signal molecules through Nrf2-Keap1 signaling pathway [30,31]. However, information regarding to the effects of tryptophan on the signal molecules Nrf2 and Keap1 in fish is lacking, and needs further investigation.

In innate immune response, pro-inflammatory cytokines and anti-inflammatory cytokines play important roles in fish and mammals [32–34].The crucial pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β), contribute to defense mechanisms of the host in response to bacterial colonization or invasion [35,36]. Conversely, the anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β) and interleukin 10 (IL-10), inhibit excessive activation of the inflammatory response [37,38]. The production of cytokines is regulated by intracellular signaling pathways of the immune system [39]. Recently, it was reported that phosphatidylinositol-4,5-bisphosphate 3-kinase/mammalian target of rapamycin (PI3K-mTOR) signaling pathway may play a critical role in innate immune system of mammals [40,41]. In myeloid phagocytes, the PI3K-mTOR pathway might serve as a decisional maker controlling the cellular response to pathogens by modulating cytokines, chemokines and type I interferon responses [42]. Inhibition of PI3K and mTOR increased the level of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) [43,44], suggesting that PI3K-mTOR pathway is a negative-feedback regulator for cytokines. In addition, in mouse bone marrow-derived macrophages (BMDM) and mDCs, the release of IL-10 was blocked after mTOR inhibition [45]. Similarly, in fish, previous studies reported that the regulation of TNF-α, IL-10 and TGF-β expression is partly by the TOR mRNA level in the head kidney of Jian carp (*Cyprinus carpio* var. Jian) [46,47]. It was also reported that the optimum tryptophan level decreased the relative gene expression of IL-8, TNF-α, while it improved IL-10, TGF-β mRNA level in grass carp (*Ctenopharyngodon idella*) [28]. Recent studies reported that the pro-inflammatory action of rapamycin is mediated by the transcription factors nuclear factor-kappa B (NF-κB), while mTOR pathway as a pivotal negative regulator of NF-κB signaling [42,43]. In human monocytes, inhibition of mTOR enhances inflammatory cytokine production via NF-κB activity [43]. The mTOR signaling plays an important role in innate immune response. However, the expression of cytokines associated with TOR signaling in fish warrants further investigation.

Blunt snout bream (*Megalobrama amblycephala*), commonly known as Wuchang bream, is a herbivorous freshwater fish species native to China. Besides, it is also distributed in Africa, North America (northern Canada to southern Mexico), Europe and other Asian countries [48]. The total output of blunt snout bream was over 0.82 million tons in 2017 [49]. Our previous research has quantified dietary tryptophan requirement of this species according to the growth performance, which was 0.20% of dry diet. We also found that tryptophan deficiency resulted in growth depression [50]. Additionally, our previous studies found that fish growth is related to its health condition [13–15,51,52]. So far, there is scared data about the impact of dietary tryptophan on antioxidant status and immunity in blunt snout bream. Therefore, in this study, we attempt to evaluate the effects of dietary tryptophan on the antioxidant capacities and immune response in the kidney of blunt snout bream.

Table 1
Ingredient and nutrient composition of experimental diets (% dry basis).

Ingredients	Diet					
	1	2	3	4	5	6
Fish meal[a]	12.00	12.00	12.00	12.00	12.00	12.00
Gelatin[a]	8.00	8.00	8.00	8.00	8.00	8.00
Carboxymethylcellulose	5.00	5.00	5.00	5.00	5.00	5.00
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00
Soybean phospholipid	3.00	3.00	3.00	3.00	3.00	3.00
Amino acid premix[b]	16.27	16.27	16.27	16.27	16.27	16.27
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin C	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin and mineral premix[c]	3.00	3.00	3.00	3.00	3.00	3.00
Mono calcium phosphate	3.00	3.00	3.00	3.00	3.00	3.00
Microcrystalline cellulose	8.07	8.07	8.07	8.07	8.07	8.07
Corn starch	35.00	35.00	35.00	35.00	35.00	35.00
Ethoxy quinoline	0.01	0.01	0.01	0.01	0.01	0.01
Glycine	0.50	0.40	0.30	0.20	0.10	0.00
L-tryptophan	0.00	0.10	0.20	0.30	0.40	0.50
Bentonite	3.00	3.00	3.00	3.00	3.00	3.00
Analyzed proximate composition						
Tryptophan (%)	0.08	0.19	0.28	0.40	0.49	0.60
Crude protein (%)	32.62	32.14	31.84	31.65	32.25	32.16
Crude lipid (%)	7.16	7.06	7.15	7.14	7.20	7.14

[a] Fish meal, obtained from obtained from Wuxi Tongwei feedstuffs Co., Ltd, Wuxi, China, crude protein 64.4%, crude lipid 9.3%; Gelatin, obtained from Wuxi Tongwei feedstuffs Co., Ltd, Wuxi, China, crude protein 91.7%.

[b] Amino acid premix (L-form, g/kg dry diet): arginine, 7.83; histidine, 4.22; Isoleucine, 10.00; leucine, 14.70; lysine, 14.00; methionine, 5.63; phenylalanine, 9.40; threonine, 8.83; valine, 9.36; aspartic acid, 16.32; serine, 8.74; glycine, 3.83; alanine, 9.20; cysteine, 1.00; tyrosine, 6.97; glutamic acid, 26.20; proline, 6.44. Amino acids obtained from Evonik Degussa Co. Ltd. (Beijing, China).

[c] Vitamins and mineral premix (IU, g or mg/kg of diet): vitamin A, 25000 IU; vitamin D3, 20000 IU; vitamin E, 200 mg; vitamin K3, 20 mg; thiamin, 40 mg; riboflavin, 50 mg; calcium pantothenate, 100 mg; pyridoxine HCl, 40 mg; cyanocobalamin, 0.2 mg; biotin, 6 mg; folic acid, 20 mg; niacin, 200 mg; inositol, 1000 mg; Vitamin C, 2000 mg; Choline, 2000 mg; 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.

2. Materials and methods

2.1. Diet preparation

Six practical diets were formulated containing graded tryptophan level (0.08%, 0.19%, 0.28%, 0.40%, 0.49% and 0.60% of dry diet). The tryptophan supplementation was replaced by an equal proportion of glycine (Table 1). Dietary protein was supplied by fish meal, corn starch and gelatins. Soybean oil and soybean lecithin were used as lipid sources. A mixture of crystalline L-amino acids was supplemented to simulate the whole-body amino acid pattern of blunt snout bream excepted for tryptophan (Table 2). The ingredients were ground into powder, which can pass through a 60-mesh sieve, and then mixed with soybean oil and water to make sinking pellet feed. The pellet feed was forced through a pelletizer (F-26 (II), South China University of Technology, China), which were then dried in a ventilated oven at 30 °C. After drying, all diets were sealed in vacuum-packed bags and stored frozen (at −20 °C) until use.

2.2. Experimental procedure

Juvenile blunt snout bream was obtained from the breeding farm of Freshwater Fisheries Research Center (FFRC) of Chinese Academy of Fishery Sciences (Wuxi, Jiangsu, China). Before the feeding trial, healthy and similar-sized fish was selected, and reared in cages

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

Amino acid	Amount in			Total	
	80 g	120 g			32% Whole
	gelatin	FM[a]	APP[b]		body protein
EAA[c]					
Arginine	0.52	0.59	0.78	1.89	1.89
Histidine	0.04	0.25	0.42	0.72	0.72
Isoleucine	0.08	0.32	1.00	1.40	1.40
Leucine	0.20	0.59	1.47	2.26	2.26
Lysine	0.24	0.65	1.40	2.29	2.29
Methionine	0.04	0.24	0.56	0.85	0.85
Phenylalanine	0.12	0.32	0.94	1.38	1.38
Threonine	0.12	0.32	0.88	1.33	1.33
Valine	0.16	0.38	0.94	1.48	1.48
Tryptophan	0.00	0.08	Variable	0.08	0.31
NEAA[d]					
Aspartic acid	0.32	0.72	1.63	2.67	2.67
Serine	0.20	0.29	0.87	1.37	1.37
Glycine	1.48	0.49	0.38	2.35	2.35
Alanine	0.60	0.49	0.92	2.01	2.01
Cystine	0.00	0.08	0.10	0.18	0.18
Tyrosine	0.04	0.27	0.70	1.01	1.01
Glutamic acid	0.68	1.07	2.62	4.37	4.37
Proline	0.84	0.32	0.64	1.81	1.81

[a] FM, fish meal.
[b] AAP, crystalline amino acid premix.
[c] EAA, essential amino acid.
[d] NEAA, non-essential amino acid.

(1 m × 1 m × 1 m). They were fed with a commercial diet containing 32% protein and 7% lipid (Wuxi Tongwei feedstuffs Co. Ltd., Wuxi, China) for two weeks to acclimatize the experimental diets and conditions. The juvenile blunt snout bream (Initial weight 3.33 ± 0.03 g) were distributed in triplicate groups of 25 fishes a cage. The fish were randomly distributed into eighteen cages and fed experimental diets three times (7:30, 12:00 and 16:00) per day for 8 weeks up to satiation on the pond culture. During the experimental period, water temperature was maintained at 28–31 °C, pH from 7.0 to 7.8, ammonia nitrogen was lower than 0.05 mg/L and dissolved oxygen was not less than 6.0 mg/L. Photoperiod was natural (light–dark cycle) throughout the experimental period.

2.3. Sample collection

At the end of the feeding trial, sampling was conducted after the fishes were fasting for 24 h from the last feeding day. Five experimental fish were collected from each cage, anesthetized with 100 mg/L MS-222. Blood samples were collected from the caudal vein with disposable medical syringes and then separated by centrifugation (3500 × *g*, 10 min, 4 °C). At the same time, kidney samples were collected from the sampled fish. Plasma and kidney samples were stored at −80 °C until analysis.

2.4. Chemical analysis

The content of lipid and protein in the experimental diets were determined in triplicate using standard methods described in Ren et al. [51]. The samples were dried in an oven at 105 °C to test moisture and were analyzed by the Kjeldahl procedure to test crude protein. Lipid content in experimental diets were analyzed by ether extraction using the Soxhelet System. Amino acid concentrations of ingredients and feeds were determined by a professional laboratory (Evonik Degussa Co. Ltd., China) as described by Ren et al. [51]. The samples were freeze-dried overnight and then hydrolyzed for 24 h in 6 N HCl at 110 °C for total amino acid contents analysis. Tryptophan content analysis was performed by hydrolyzed for 20 h in 5 N NaOH at 110 °C.

Table 3
Primer sequence for qRT-PCR.

Target gene		Primer sequence (5′-3′)	Amplicon length (bp)
TOR[a]	Forward	TTTACACGAGCAAGTCTACGGA	22
	Reverse	CTTCATCTTGGCTCAGCTCTCT	22
PI3K[b]	Forward	AAGAAAGTTTGCCACACCGC	20
	Reverse	TTGTCCATGGTTCAGTGGCA	20
Akt[c]	Forward	GCTGGGTAAAGGCACGTTTG	20
	Reverse	CTCTCGGTGACCGTATGAGC	20
NF-κB[d]	Forward	AGTCCGATCCATCCGCACTA	20
	Reverse	ACTGGAGCCGGTCATTTCAG	20
Nrf2[e]	Forward	GGGGAAGTCCTTGAACGGAG	20
	Reverse	AACCAGCGGGAATATCTCGG	20
Keap1[f]	Forward	AATATCCGCCGGCTGTGTAG	20
	Reverse	TGAGTCCGAGGTGTTTCGTG	20
CAT[g]	Forward	CAGTGCTCCTGATACCCAGC	20
	Reverse	TTCTGACACAGACGCTCTCG	20
GPx[h]	Forward	GAACGCCCACCCTCTGTTTG	20
	Reverse	CGATGTCATTCCGGTTCACG	20
Cu/Zn-SOD[i]	Forward	AGTTGCCATGTGCACTTTTCT	21
	Reverse	AGGTGCTAGTCGAGTGTTAGG	21
Mn-SOD[j]	Forward	AGCTGCACCACAGCAAGCAC	20
	Reverse	TCCTCCACCATTCGGTGACA	20
IL-8[k]	Forward	CAGAGAGTCGACGCATTGGT	20
	Reverse	ATTCACGGTGCTTTGTTGGC	20
IL-10[l]	Forward	GTGTTTTCGGGTGCAAGTGG	20
	Reverse	ATGAACGAGATCCTGCGCTT	20
IL-1β[m]	Forward	TTCTTCCCCTCACCTGGTCT	20
	Reverse	CCAGCGCGAAGTTTGTCAAT	20
TNF-α[n]	Forward	TGGAGAGTGAACCAGGACCA	20
	Reverse	AGAGACCTGGCTGTAGACGA	20
TGF-β[o]	Forward	ACTGGACAAACAGAGAGGCG	20
	Reverse	CAGGGGAGTTGCCGTTAGAG	20
HO-1[p]	Forward	TCACACCGGGAAACGAGAAG	20
	Reverse	TGGAGCATTTCTACGGCCAG	20
β-actin	Forward	TCGTCCACCGCAAATGCTTCTA	22
	Reverse	CCGTCACCTTCACCGTTCCAGT	22

[a] TOR, target of rapamycin.
[b] PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase.
[c] Akt, protein kinase B (pkb, also known as Akt).
[d] NF-κB, nuclear factor-kappa B.
[e] Nrf2, nuclear factor erythroid 2-related factor 2.
[f] Keap1, Kelch-like ECH-associated protein 1.
[g] CAT, catalase.
[h] GPx, glutathione peroxidase.
[i] Cu/Zn-SOD, copper zinc superoxide dismutase.
[j] Mn-SOD, manganese superoxide dismutase.
[k] IL-8, interleukin 8.
[l] IL-10, interleukin 10.
[m] IL-1β, interleukin 1β.
[n] TNF-α, tumour necrosis factor-α.
[o] TGF-β, transforming growth factor-β.
[p] HO-1, heme oxygenase-1.

Plasma albumin (ALB), alanine transaminase (ALT), aspartate aminotransferase (AST), complement component 3 (C3), immune globulin M (IgM) and alkaline phosphatase (ALP) were tested by a Mindray BS-400 automatic biochemical analyzer (Mindray Medical International Ltd., Shenzhen, China). Assay kits were purchased from Shenzhen Mindary Bio-medical Electronics Co. Ltd (Shenzhen, China).

Plasma contents of malondialdehyde (MDA), total superoxide dismutase (T-SOD), catalase (CAT), glutathione peroxidase 1 (GPx-1), total antioxidant capacity (T-AOC) and glutathione (GSH) were assayed by using assay kits (Jian Cheng Bioengineering Institute, Nanjing, China).

Relative gene expressions of antioxidant and immunity were determined by using Real-time PCR analysis. Firstly, total RNA was extracted from the kidney of juvenile blunt snout bream using an RNAiso plus kit (Takara, Dalian, China). After quality and quantity of RNA were checked, complementary DNA (cDNA) was synthesized using a PrimeScript™ RT reagent kit (Takara, Dalian, China). Specific primers

Table 4
Effects of dietary tryptophan levels on plasma parameters of blunt snout bream (Means ± SEM.)[a].

Tryptophan	AST[b]	IgM[c]	ALP[d]	C3[e]	ALT[f]	ALB[g]
level (%)	(U/L)	(mg/L)	(U/L)	(mg/L)	(U/L)	(g/L)
0.08	102.48 ± 5.04[b]	11.01 ± 0.96[a]	43.18 ± 3.51[a]	26.25 ± 0.51	5.29 ± 0.83	11.23 ± 0.33
0.19	69.55 ± 3.88[a]	12.806 ± 1.22[a]	58.37 ± 6.24[ab]	27.51 ± 0.61	4.63 ± 0.56	12.53 ± 0.72
0.28	63.99 ± 5.06[a]	22.65 ± 1.03[b]	61.07 ± 4.68[ab]	28.00 ± 0.41	3.68 ± 0.47	12.63 ± 0.53
0.40	69.26 ± 3.20[a]	23.69 ± 2.21[b]	72.07 ± 8.09[b]	27.87 ± 0.41	3.69 ± 0.49	13.16 ± 0.45
0.49	73.17 ± 5.42[a]	22.51 ± 1.47[b]	64.48 ± 6.12[ab]	27.67 ± 0.49	4.86 ± 0.61	11.90 ± 0.37
0.60	75.07 ± 7.86[a]	21.25 ± 1.92[b]	53.94 ± 4.96[ab]	27.37 ± 0.60	6.04 ± 0.70	11.97 ± 0.55

[a] Data are means of triplicate; means in the same column sharing the same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).
[b] AST, aspartate transaminase.
[c] IgM, immune globulin M.
[d] ALP, alkaline phosphatase.
[e] C3, complement component 3.
[f] ALT, alanine aminotransferase.
[g] ALB, albumin.

for relative genes were shown in Table 3. β-actin was employed as a nonregulated reference gene. No significant changes were found in the present study or our previous studies [13,52,53]. Relative quantification of target gene expression was performed using the Pfaffl's mathematical model [54].

2.5. Statistics analysis

Data were subjected to one-way analysis of variance (ANOVA) using the SPSS software 16.0 for Windows. Significant differences between means were evaluated by Tukey's Test. Probabilities of $P < 0.05$ were considered significant. Data are presented as the means ± SEM.

3. Results

3.1. Plasma parameters

The results of plasma parameters were shown in Table 4. The aspartate transaminase (AST) activity in 0.08% group was strikingly higher than other groups ($P < 0.05$). Immune globulin M (IgM) and alkaline phosphatase (ALP) in 0.40% group were the highest, and significantly higher than those in fish fed with 0.08% tryptophan diet ($P < 0.05$). The activity of alanine aminotransferase (ALT), albumin (ALB) and complement component 3 (C3) were trivially affected by increasing dietary tryptophan level ($P > 0.05$).

The results of plasma antioxidant enzymes were shown in Table 5. Total superoxide dismutase (T-SOD), catalase (CAT), total antioxidant capacity (T-AOC) activity were significantly increased with increasing dietary tryptophan level until up to 0.40%, and then decreased ($P < 0.05$). The activity of T-SOD, T-AOC and glutathione (GSH) in 0.40% group were significantly higher than those in fish fed with 0.08% tryptophan diet ($P < 0.05$). However, malondialdehyde (MDA) content showed a reverse trend compared with T-SOD ($P < 0.05$). 0.19% and 0.28% dietary tryptophan level significantly improved the plasma glutathione peroxidase 1 (GPx-1) activity compared with 0.08% and 0.60% tryptophan level ($P < 0.05$). Based on MDA and T-SOD, the optimal dietary tryptophan level was determined to be 0.33% (1.03% of dietary protein) and 0.36% (1.13% of dietary protein), respectively, using quadratic regression analysis (Fig. 1; Fig. 2).

3.2. The relative expression of antioxidant genes

The results of the relative expression level of antioxidant genes were shown in Fig. 3. The relative gene expression level of nuclear factor erythroid 2-related factor 2 (Nrf2), glutathione peroxidase (GPx) and manganese superoxide dismutase (Mn-SOD), copper-zinc superoxide dismutase (Cu/Zn-SOD), catalase (CAT) and heme oxygenase-1 (HO-1) were significantly up-regulated with increasing dietary tryptophan level up to a certain level ($P < 0.05$). Nrf2, GPx and Mn-SOD mRNA level in 0.40% group were significantly higher than those in fish fed 0.08% tryptophan diet ($P < 0.05$). Compared with 0.60% tryptophan level, 0.28% dietary tryptophan level significantly increased the relative expression of Cu/Zn-SOD and HO-1 ($P < 0.05$). While the relative expression of Keap1 was significantly decreased with increasing dietary tryptophan level until up to 0.40%, and then increased ($P < 0.05$).

Table 5
Effects of dietary tryptophan levels on plasma antioxidant enzymes of blunt snout bream (Means ± SEM.)[a].

Tryptophan	MDA[b]	T-SOD[c]	GSH[d]	GPx-1[e]	CAT[f]	T-AOC[g]
level (%)	(nmol/ml)	(U/ml)	(umol/L)	(IU)	(U/ml)	(Unit/ml)
0.08	14.50 ± 0.83[b]	37.66 ± 4.94[a]	127.92 ± 15.46[a]	145.98 ± 13.43[a]	2.35 ± 0.29[ab]	3.07 ± 0.24[a]
0.19	13.03 ± 0.92[ab]	53.51 ± 4.21[ab]	122.16 ± 16.03[a]	183.86 ± 12.95[b]	2.69 ± 0.25[ab]	3.24 ± 0.26[ab]
0.28	11.72 ± 0.57[a]	59.74 ± 3.71[ab]	132.67 ± 8.03[ab]	174.45 ± 9.17[b]	3.31 ± 0.21[ab]	3.55 ± 0.39[ab]
0.40	12.93 ± 0.77[ab]	61.52 ± 4.44[b]	158.44 ± 12.97[b]	163.89 ± 8.70[ab]	3.48 ± 0.37[b]	4.53 ± 0.35[b]
0.49	13.83 ± 0.72[b]	46.44 ± 5.99[ab]	151.99 ± 16.00[b]	157.92 ± 13.89[ab]	3.06 ± 0.17[ab]	3.65 ± 0.34[ab]
0.60	14.00 ± 0.68[b]	48.14 ± 6.35[ab]	134.09 ± 13.74[ab]	143.57 ± 11.43[a]	2.16 ± 0.46[a]	3.10 ± 0.22[a]

[a] Data are means of triplicate; means in the same column sharing the same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).
[b] MDA, malondialdehyde.
[c] T-SOD, total superoxide dismutase.
[d] GSH, glutathione.
[e] GPx-1, glutathione peroxidase 1.
[f] CAT, catalase.
[g] T-AOC, total antioxidant capacity.

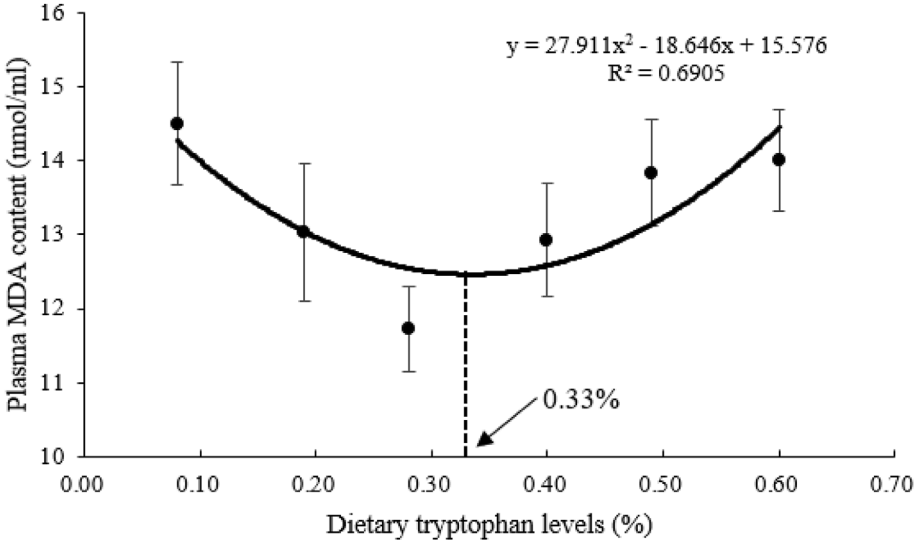

Fig. 1. Quadratic regression analysis of malondialdehyde (MDA, nmol/ml) against varying dietary tryptophan levels of blunt snout bream.

3.3. The relative expression of immune genes

The results of the relative expression of immune genes were shown in Fig. 4. The relative expression of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), protein kinase B (Akt) and target of rapamycin (TOR) were strikingly up-regulated with increasing dietary tryptophan level up to a certain level, and then decreased ($P < 0.05$). Compared with 0.08% and 0.60% tryptophan level, 0.40% dietary tryptophan level significantly improved transforming growth factor-β (TGF-β) mRNA levels ($P < 0.05$). 0.28% dietary tryptophan level contributed to improve relative expression level of interleukin 10 (IL-10), compared with 0.08% and 0.49% level ($P < 0.05$). The relative gene expression level of nuclear factor-kappa B (NF-κB), tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) were significantly down-regulated with increasing of dietary tryptophan levels up to a certain level, and then increased ($P < 0.05$). The mRNA level of NF-κB, TNF-α and IL-1β in 0.40% group were significantly lower than those in 0.08% group ($P < 0.05$). The relative gene expression of interleukin 8 (IL-8) showed a similar trend as TNF-α and IL-1β. However, IL-8 mRNA levels were independent of tryptophan supplementation ($P > 0.05$).

4. Discussion

It is known that a balanced nutrition intake is essential not only improving growth for aquatic animals, but also maintaining their health. For juvenile growing stages, the nutritional imbalance has a harmful effect on growth performance and may even lead to disease [12]. Our previous study has been reported that proper dietary tryptophan level could improve the growth performance of juvenile blunt snout bream [50]. The current study indicated that the tryptophan level was also closely related to the health of blunt snout bream. Plasma aspartate aminotransferase (AST) and alanine transaminase (ALT) are used to measure tissue damage [55]. In this study, 0.08% dietary tryptophan contributed to higher AST and ALT activity, which indicated that dietary tryptophan imbalance weakened liver function of juvenile blunt snout. In this kind of fish, arginine imbalance also damages their liver function [52]. The complement system is a humoral defense component in teleost, and complement component 3 (C3) plays a crucial role in the activation of complement system [56,57]. In this study, plasma C3 content trivially increased with increasing dietary tryptophan level, and lower plasma C3 level was found in tryptophan deficiency diet. Similar result was also reported in threonine on blunt

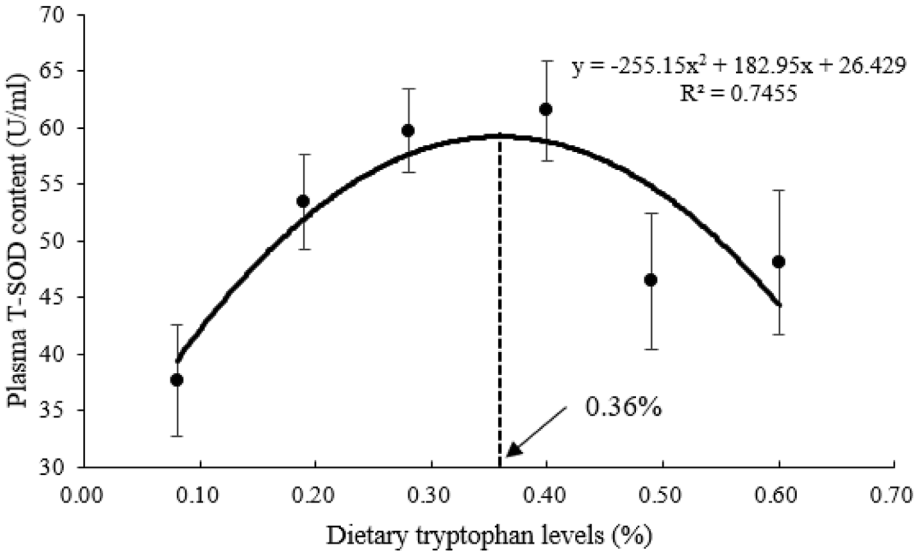

Fig. 2. Quadratic regression analysis of total superoxide dismutase (T-SOD, U/ml) against varying dietary tryptophan levels of blunt snout bream.

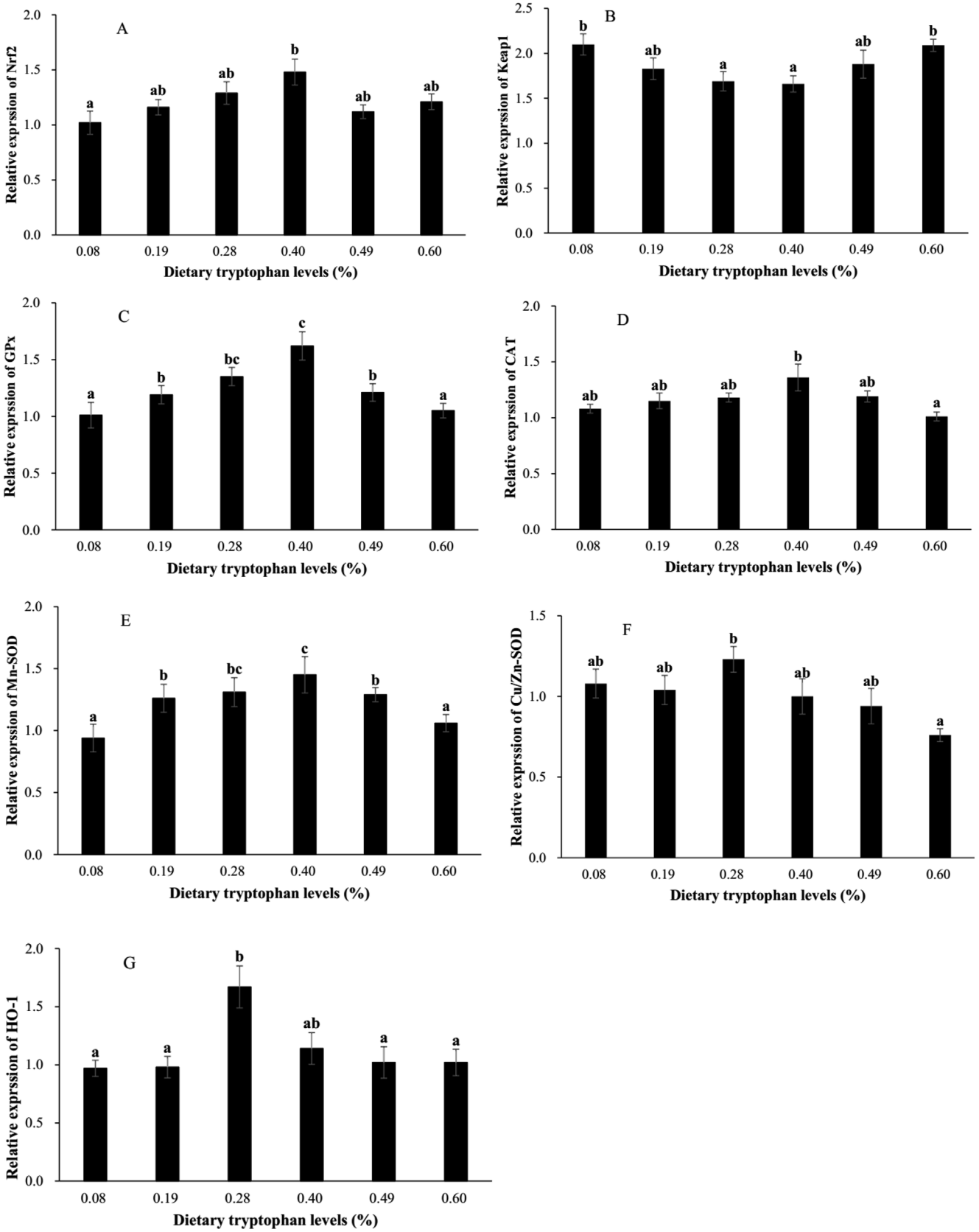

Fig. 3. Antioxidant-related parameters gene expressions in kidney of blunt snout bream: (A) nuclear factor erythroid 2-related factor 2 (Nrf2); (B) Kelch-like ECH-associated protein 1 (Keap1); (C) glutathione peroxidase (GPx); (D) catalase (CAT); (E) manganese superoxide dismutase (Mn-SOD); (F) copper zinc superoxide dismutase (Cu/Zn-SOD); (G) heme oxygenase-1 (HO-1). Data are expressed as means ± SEM. Mean values of graded dietary tryptophan levels of each parameter with unlike small letters above bars are significantly different ($P < 0.05$).

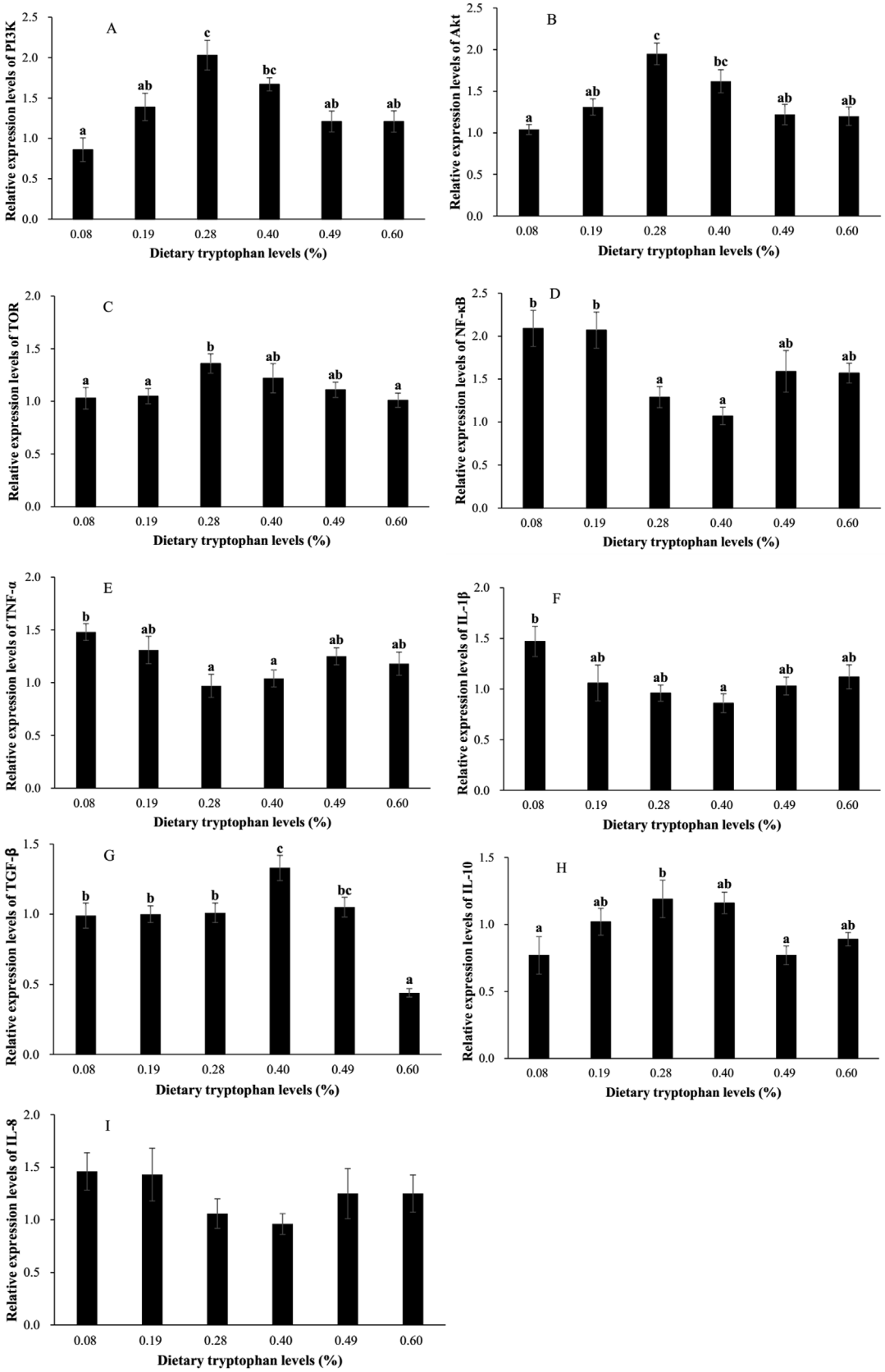

Fig. 4. Relative expression levels of cytokines genes in kidney of blunt snout bream: (A) phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K); (B) protein kinase B (Akt); (C) target of rapamycin (TOR); (D) nuclear factor-kappa B (NF-κB); (E) tumour necrosis factor-α (TNF-α); (F) interleukin 1β (IL-1β); (G) transforming growth factor-β (TGF-β); (H) interleukin 10 (IL-10); (I) interleukin 8 (IL-8). Data are expressed as means ± SEM. Mean values of graded dietary tryptophan levels of each parameter with unlike small letters or big letters above bars are significantly different ($P < 0.05$).

snout bream [14]. Immunoglobulin producing is also an adaptive response after being stimulated by antigen in fish, and immune globulin M (IgM) class is the primary immunoglobulin in most teleost [58]. In our study, 0.28% or more tryptophan level led to the increased production of IgM than tryptophan deficiency diet. Similar results were found in both pigs and fish that adequate threonine or leucine intake improved plasma immune globulin G (IgG) or IgM level to enhance immunologic defense [14,15,59]. Our present study suggested that adequate tryptophan could improve serum parameters related to immune response to resist damage in fish.

Increasing studies showed that optimal tryptophan supplement could improve the antioxidant status in animals [30,31]. In previous studies, the plasma contents of antioxidant enzymes and non-antioxidant enzymes are used to determine the antioxidant status [20,60,61]. Malondialdehyde (MDA), a kind of lipid peroxide, which reflects the body's peroxidation [62] and can also indirectly reflect the cell damage. In this study, 0.28% dietary tryptophan level significantly decreased the plasma MDA content, which means tryptophan inhibits the lipid peroxidation of blunt snout bream. This result was in agreement with grass carp (*Ctenopharyngodon idella*) [28]. However, the study of whether tryptophan could improve antioxidation of fish is scared. In rat, the study showed that tryptophan remarkably decreased the MDA content in plasma [63]. Our result indicated that the optimal dietary tryptophan level could protect fish against oxidative damage. Glutathione (GSH) is not only a non-antioxidant enzyme, but also a low molecular remover which can remove O_2^- and H_2O_2. In our study, with the increasing dietary tryptophan level, the GSH content increased, and had the highest value in 0.40% tryptophan level, which means tryptophan could improve the non-antioxidant status of fish. Raju et al. [64] reported that tryptophan supplement could increase GSH content of the liver in rat. Other amino acids, such as histidine and lysine also could increase the intestinal GSH content [65,66]. Superoxide dismutase (SOD) and glutathione peroxidase 1 (GPx-1) are important antioxidant enzymes [67]. Catalase (CAT) is an essential defense against the potential toxicity of free radical like hydroxyl radical by catalyzing the degradation of hydrogen peroxide [68]. In this study, 0.40% dietary tryptophan significantly improved the activity of T-SOD, GPx-1 and CAT, which means tryptophan could improve the activity of antioxidant enzymes of blunt snout bream. This result was consistent with tryptophan on grass carp [28], as well as threonine, leucine and arginine on blunt snout bream [14,15,52]. Total antioxidant capacity (T-AOC) directly reflected the antioxidant capacity of fish [69]. In this study, T-AOC activity was significantly improved by 0.40% tryptophan level. These results indicated that optimal tryptophan level has the protective effects on oxidative damage due to the increased enzymatic antioxidant capacity and non-enzymic defense in blunt snout bream.

Proper nutrition is critical not only to achieve optimal growth rates but also to maintain the health of cultured fish. Nowadays, the definition of “immunonutrition” was appeared, which could be higher than those level needed for optimal growth [12,14]. In our previous study, it has been reported that optimal tryptophan could improve the growth performance of juvenile blunt snout bream, and the tryptophan requirement was quantified as 0.20% based on the growth performance [50]. In the current study, based on MDA and T-SOD level, the optimal dietary tryptophan level was determined to be 0.33% (1.03% of dietary protein) and 0.36% (1.13% of dietary protein), which was higher than the requirement we previously determined. This result was consistent with the enzymes above, and the possible explanation is that fish need better dietary nutrition to improve health status compared to maintain growth performance [12]. And for blunt snout bream, we also found similar tends in threonine, arginine, leucine and histidine [13–15,51–53,70,71].

The previous studies have reported that the production of antioxidant enzymes is related to their corresponding gene expression [13–15,29]. Studies have shown that nuclear factor erythroid 2-related factor 2 (Nrf2) is an important molecule in the regulation of antioxidant enzyme-related gene expression in fish, and the Nrf2-Keap1 complex blocked the translocation of Nrf2 [15,27,72]. In the present study, Keap1 was significantly decreased with increasing dietary tryptophan level up to 0.40%, and then increased, while Nrf2 showed an opposite tendency. This result was consistent with tryptophan on juvenile grass carp [28]. In addition, 0.40% tryptophan level improved manganese superoxide dismutase (Mn-SOD), CAT and GPx gene expression level in the kidney of juvenile blunt snout bream significantly, which was corresponding with the activity of plasma CAT, GPx-1 and T-SOD. Previous studies also reported that the increase in antioxidant enzyme activity is related to its corresponding gene expression level, and its gene expression level is related to the Nrf2/Keap1 signaling pathway [14,15,29]. Similar results were found on threonine, arginine and leucine [14,15,52]. Xu et al. [73] also found that the enhancement of Nrf2 nuclear translocation can promote the mRNA level of antioxidant enzyme-related genes in human prostate cells. This explained why tryptophan promoted the activity of SOD and GPx-1, which may be due to tryptophan up-regulated the expression level of corresponding genes. Heme oxygenase 1 (HO-1), the rate-limiting enzyme of heme catabolism [74], is a cellular defense component opposed to oxidative stress [75], which is associated with Nrf2 [73,76]. The present study showed that 0.28% tryptophan level up-regulated the relative expression of HO-1, which was in agreement with our previous study in leucine [15]. Similarly, in human prostate cancer PC-3 cells, nuclear Nrf2 activates expression of stress-responsive genes [73]. In mouse and human PC12 cells, the improvement of HO-1 activity and expression could protect cells and tissues from subsequent injuries [75,77]. The present results indicated that optimal dietary tryptophan could promote the antioxidant status of juvenile blunt snout bream by regulating Nrf2/Keap1 signaling pathway and its corresponding antioxidant enzyme genes.

Mammalian target of rapamycin (mTOR) signaling is sensitive to the availability of amino acids in terrestrial animal [78]. Recently, emerging evidence indicated that mTOR signaling plays a pivotal role in the immune system on monocytes and DCs [79], and can mediated the transcription of cytokine in murine and human immune cells [42]. Previous studies showed that dietary amino acids could regulate the relative gene expressions of TOR in fish [13,28,46,80]. In our study, dietary tryptophan also regulated TOR expression. It showed that TOR mRNA levels were significantly increased with dietary tryptophan levels up to 0.28%, and then decreased. The current result was consistent with the results in isoleucine on grass carp [46]. On Jian carp and grass carp, it was reported that dietary tryptophan could affect TOR mRNA levels as well [4,28]. In myeloid phagocytes, the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)–mTOR pathway might be a decisional maker controlling the cellular response to pathogens by modulating cytokines, chemokines and type I interferon responses [42]. The result of a study on a murine cecal ligation and puncture-induced polymicrobial sepsis model supporting that the PI3K pathway is a negative inflammatory feedback regulator for cytokines [81]. In this study, PI3K and protein kinase B (Akt) mRNA level were strikingly up-regulated in 0.28% diet, which was consistent with TOR. Moreover, the mRNA level of tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β) and interleukin 8 (IL-8) in kidney of juvenile blunt snout bream decreased as the dietary tryptophan level increased up to a certain level. On grass carp, the supplement of tryptophan decreased the mRNA level of IL-8 and TNF-α in intestine [28]. As for other amino acids, for example leucine, could also down-regulate the expression of TNF-α, IL-1β and IL-8 [15]. Hu et al. [82] also found that dietary glutamine supplementation down-regulated the IL-1β and IL-10 gene expression of Jian carp. In blunt snout bream, our previous study found that optimal arginine strikingly down-regulated the expression of TNF-α and IL-1β, and increased transforming growth factor-β (TGF-β) expression level in intestine [52]. In the liver, certain dietary leucine and threonine decreased the TNF-α mRNA level [14,15]. In murine, pharmacological inhibition of PI3K, led to the amplified production of IL-1β, interleukin 6 (IL-6), interleukin 12

(IL-12), and TNF-α [83]. Studies in DCs reported that the inhibition of mTOR weakens the global inflammatory response [83,84]. In addition, nuclear factor-kappa B (NF-κB) might be involved in inflammation response [40], and can mediate the proinflammatory action of rapamycin [42]. In the present study, 0.28% and 0.40% diet inhibited NF-κB mRNA level, which was consistent with the expression of TNF-α, IL-1β and IL-8. Studies in arginine on blunt snout bream and grass carp also found similar results [52,85]. In human monocytes, it was found that the transcription factor NF-κB is a master regulator of pro-inflammatory response and is also a major regulator of IL-12p40, and mTOR negatively regulates NF-κB [43]. According to the studies above, we speculated that tryptophan may control inflammatory cytokines through PI3K-TOR and NF-κB pathway. However, the information about how tryptophan affects immune system is lacking, and the underlying mechanism that tryptophan attenuates the inflammation response via the TOR signaling pathway needs further investigation.

In conclusion, based on MDA and T-SOD level, the optimal dietary tryptophan level was 0.33% dry diet (1.03% of dietary protein) and 0.36% dry diet (1.13% of dietary protein) for juvenile blunt snout bream. The optimal dietary tryptophan level could improve plasma antioxidant enzymes activity and up-regulate their gene transcriptions in kidney, which might be regulated by Nrf2/Keap1 signaling pathway. Furthermore, the optimal dietary tryptophan level could improve immunocompetence in juvenile blunt snout bream by down-regulating pro-inflammatory genes level while up-regulating anti-inflammatory genes mRNA level via PI3K-TOR and NF-κB in kidney.

Acknowledgments

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