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Effects of dietary protein levels on the disease resistance, immune function and physical barrier function in the gill of grass carp (*Ctenopharyngodon idella*) after challenged with *Flavobacterium columnare*

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ABSTRACT

The effects of dietary protein levels on the disease resistance, gill immune function and physical barrier function of grass carp (*Ctenopharyngodon idella*) were investigated in this study. A total of 540 grass carp (264.11 ± 0.76 g) were fed six diets containing graded levels of protein (143.1, 176.7, 217.2, 257.5, 292.2 and 322.8 g digestible protein kg^{-1} diet) for 8 weeks. After the growth trial, fish were challenged with *Flavobacterium columnare* for 3 days. The results indicated that optimal levels of dietary protein had the following effects: (1) the production of antibacterial components increased, and anti-inflammatory cytokines, inhibitor of κBα, target of rapamycin and ribosomal protein S6 kinases 1 mRNA levels were up-regulated, whereas mRNA levels of pro-inflammatory cytokines, nuclear factor kappa B (NF-κB) P65, NF-κB P52, IκB kinase (IKK) α, IKKβ, IKKγ, eIF4E-binding proteins (4E-BP) 1 and 4E-BP2 were down-regulated in the gills of grass carp ($P < 0.05$), indicating that fish gill immune function was enhanced at an optimal level of dietary protein; (2) the activities and mRNA levels of antioxidant enzymes and glutathione content increased, the contents of reactive oxygen species, malondialdehyde and protein carbonyl (PC) decreased, and NF-E2-related factor 2, B-cell lymphoma protein-2, inhibitor of apoptosis proteins, myeloid cell leukemia-1 and tight junction complexes mRNA levels were up-regulated, whereas Kelch-like-ECH-associated protein (Keap) 1a, Keap1b, cysteinyl aspartic acid-protease 3, 8, 9, fatty acid synthetase ligand, apoptotic protease activating factor-1, Bcl-2 associated X protein, c-Jun N-terminal protein kinase, myosin light chain kinase and p38 mitogen-activated protein kinase mRNA levels were down-regulated in the gills of grass carp ($P < 0.05$), indicating that the fish gill physical barrier function improved at an optimal level of dietary protein. Finally, based on the gill rot morbidity, ACP activity and PC content, the optimal levels of dietary protein for grass carp were estimated to be 286.65 g kg^{-1} diet (253.73 g digestible protein kg^{-1} diet), 290.46 g kg^{-1} diet (257.76 g digestible protein kg^{-1} diet) and 296.25 g kg^{-1} diet (260.69 g digestible protein kg^{-1} diet), respectively.

1. Introduction

The fish gill is an essential immune-competent organ that plays a crucial role in respiration, homeostatic equilibrium and immune response [1]. Studies demonstrate that disturbance to fish gills leads to a decline in growth [2] and even an increase in the mortality rate [3]. Therefore, maintaining the status of gill health is

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critically important to fish. In our previous study, dietary protein supplementation improved the growth performance of grass carp [4]. It was reported that fish growth is positively correlated with gill health status [2]. In fish, gill health status is largely dependent on its immune function [5] and physical barrier function [6]. Studies in our laboratory show that nutrients such as riboflavin [7] and folic acid [8] improve the gill immune function and physical barrier function of grass carp (*Ctenopharyngodon idellus*). Seifter et al. [9] found that the content of riboflavin in the liver of rats decreased with a low protein diet. These data suggest that dietary protein levels may be associated with fish gill immune function and physical barrier function, and therefore, these relations warrant investigation.

To our knowledge, gill immune function depends in part on the immune response in fish [5]. According to one report, antibacterial compounds and cytokines play crucial roles in the immune responses of fish [10]. The transcription levels of cytokines in humans are mediated by nuclear factor kappa B (NF-κB) [11] and target of rapamycin (TOR) [12] signalling pathways. However, studies have not investigated the effects of dietary protein level on gill immune function or on the possible mechanisms for such effects on fish. A report demonstrated that supplementation with protein increases serum levels of insulin-like growth factor 1 (IGF-1) in humans [13], and Pelosi et al. [14] demonstrated that up-regulation of IGF-1 led to the down-regulation of gene expression of cytokines tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in mice skeletal muscle. Montaseri et al. [15] found that IGF-1 suppresses NF-κB activation in human articular chondrocytes, and Xian et al. [16] indicated that IGF-1 activates TOR in mice mesenchymal stem cells. Based on these studies, a relationship between dietary protein levels and gill immune function and the related signalling pathways in fish is apparent, which is worthy of investigation.

In addition to the immune function of the gill, the physical barrier function also plays an important role in maintaining the gill structural integrity of fish, with the barrier composed of epithelial cells [17] and intercellular tight junction complexes (TJs) [18]. Based on our understanding, the disruption of gill epithelial cells is highly correlated with oxidative damage [19] and apoptosis [20] in fish. According to reports, the transcript abundance related to antioxidants and apoptosis may be regulated by NF-E2-related factor 2 (Nrf2) [21] and c-Jun N-terminal protein kinase (JNK) [22] in fish, respectively. However, to date, no reports have focused on the effects of dietary protein levels on the structural integrity of gill epithelial cells or on the possible mechanisms for such effects in fish. In humans, dietary protein supplementation increases glucagon-like peptide 1 (GLP-1) [23] and ghrelin concentration [24]. Abdi et al. [25] found that GLP-1 increases the transcription of Nrf2 in humans, and Wang et al. [26] reported that ghrelin inhibits apoptosis and JNK activity in MIN6 cells. Based on these data, a possible relationship between dietary protein levels and antioxidant capacity and apoptosis and the related signalling pathways in fish gills are likely, which is worthy of further investigation. Meanwhile, the tight junction complexes (TJs) are essential components of the physical barrier, which includes occludin, zonula occludens (ZO) and claudins in the gills of fish [27]. In fish, the gene expression of TJs is regulated by myosin light chain kinase (MLCK) [28]. However, the effects of dietary protein levels on the TJs of gills or the possible mechanism for such effects in fish have not been addressed in any study. In rats, dietary protein supplements increase the absorption of intestinal iron [29], and Wang et al. [30] observed that iron supplementation led to up-regulated mRNA levels of occludin in the brain of rats. In chicks, a low-protein diet elevates the plasma cholesterol level [31], and Zhu et al. [32] demonstrated that a high cholesterol level increases MLCK expression in rabbits. According to these data, a correlation between dietary protein levels and the TJs of fish gills and the related signalling pathway is possible, which requires further investigation.

In this study, the growth trial was identical to that used in our previous study [4], which is part of a larger research effort to determine the effects of dietary protein levels on fish growth and health status. Because a report indicates that the growth is also closely correlated with the health of gills in fish [2], we hypothesized that the optimal level of dietary protein would increase gill immune function and physical barrier function and thereby improve the status of fish gill health. To test the hypothesis and to first identify the gill immune function and physical barrier function of fish, we investigated the effects of dietary protein levels on antibacterial components, cytokines, antioxidants, apoptosis and intercellular TJs in the gills of grass carp after challenge with *Flavobacterium columnare*. Additionally, we further investigated the effects of dietary protein levels on the related signalling molecules that included NF-κB, TOR, Nrf2, JNK and MLCK in the gills of grass carp because an effect on these signalling molecules would provide theoretical evidence, in part, for the mechanisms of protein-regulated fish gill immune function and physical barrier function to maintain gill health. Meanwhile, the optimal levels of dietary protein for grass carp based on different indices were also evaluated, which may provide a practical evidences for the protective effects of dietary protein levels on the gill health of fish.

2. Materials and methods

2.1. Experimental diet and procedures

As shown in Table 1, the formulation and approximate composition analysis of the diets are the same as our previous study [4]. According to Deng et al. [33], fish meal, casein and gelatin were used as dietary protein sources in a particular ratio at 6:16:3. Crystalline amino acid mixtures were supplemented to simulate the amino acid pattern according to the method described by Wang et al. [34] and Gao et al. [35]. Fish oil and soybean oil were used as dietary lipid sources. Six experimental diets with different protein levels (170.0, 210.0, 250.0, 290.0, 330.0 and 370.0 g kg^{-1} diet) were used. The diets were formulated to be iso-energetic according to the method of Garling et al. [36]. According to Kjeldahl method, protein contents in the experimental diets were measured to be 169.2, 204.7, 244.3, 283.2, 323.3 and 366.3 g kg^{-1} diet. The digestible protein levels of grass carp in the six experimental diets were estimated to be 143.1, 176.7, 217.2, 257.5, 292.2 and 322.8 g kg^{-1} diet, respectively (according to our previous study [4]). After being prepared completely, the diets were stored at −20 °C as described by Takakuwa et al. [37].

2.2. Growth trial

The procedures used in this study were approved by the Animal Care Advisory Committee of Sichuan Agricultural University. Grass carp were obtained from fisheries (Sichuan, China). Before starting the experiment, fish were acclimated to the experimental environment for 4 weeks according to Kpogue et al. [38]. Then, 540 fish (mean weight 264.11 ± 0.76 g) were randomly assigned to 18 experimental cages (1.4 L × 1.4 W × 1.4 H m), resulting in 30 fish per cage as described in our laboratory study [39]. Every cage was equipped with a disc of 100 cm diameter in the bottom to collect the uneaten feed according to our laboratory study [40]. For the feeding trial, fish were fed with their respective diets four times daily for 8 weeks according to Hossain et al. [41]. Thirty minutes after feeding, uneaten feed was collected, dried and weighed to calculate the feed intake according to our laboratory study [42].

Table 1
Composition and nutrients content of basal diet.

Ingredients	Dietary protein levels (dietary digestible protein levels) (g kg^{-1})					
	169.2 (143.1)	204.7 (176.7)	244.3 (217.2)	283.2 (257.5)	323.2 (292.2)	366.3 (322.8)
Fish meal	50.1	62.2	74.3	86.5	98.6	110.8
Casein	133.5	165.9	198.2	230.7	263.0	295.5
Gelatin	25.0	31.1	37.2	43.3	49.3	55.4
α-starch	240.0	240.0	240.0	240.0	240.0	240.0
Corn starch	402.6	352.1	301.8	251.2	201.0	150.5
Fish oil	7.9	7.1	6.3	5.5	4.7	3.9
Soybean oil	19.2	19.2	19.2	19.2	19.2	19.2
Microcrystalline cellulose	50.0	50.0	50.0	50.0	50.0	50.0
$Ca(H_2PO_4)_2$	33.0	33.0	33.0	33.0	33.0	33.0
DL-Met (98%)	1.7	2.1	2.4	2.7	3.1	3.3
L-Arg (99%)	1.5	1.8	2.1	2.4	2.6	2.9
Vitamin premix[a]	10.0	10.0	10.0	10.0	10.0	10.0
Mineral premix[b]	20.0	20.0	20.0	20.0	20.0	20.0
Choline chloride (60%)	5.0	5.0	5.0	5.0	5.0	5.0
Ethoxyquin (30%)	0.5	0.5	0.5	0.5	0.5	0.5
Nutrient contents						
Moisture[c]	137.0	139.7	142.5	143.5	138.7	131.8
Crude protein[c]	169.2	204.7	244.3	283.2	323.2	366.3
Digestible protein[c]	143.1	176.7	217.2	257.5	292.2	322.8
Crude lipid[c]	33.1	32.5	32.2	32.2	33.0	33.1
n-3[d]	5.0	5.0	5.0	5.0	5.0	5.0
n-6[d]	10.0	10.0	10.0	10.0	10.0	10.0

[a] Per kilogram of vitamin premix (g kg^{-1}): retinyl acetate (500,000IU/g), 2.10; cholecalciferol (500,000IU/g), 0.40; D, L-α-tocopherol acetate (50%), 12.58; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.11; ascorhyl acetate (95%), 4.31; niacin (99%), 2.58; meso-inositol (98%), 19.39; calcium-D-pantothenate (98%), 2.56; riboflavin (80%), 0.63; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with corn starch to 1 kg.
[b] Per kilogram of mineral premix (g kg^{-1}): $MnSO_4.H_2O$ (31.8% Mn), 1.8900; $MgSO_4 \cdot H_2O$ (15.0% Mg), 200.0000; $FeSO_4.H_2O$ (30.0% Fe), 24.5700; $ZnSO_4.H_2O$ (34.5% Zn), 8.2500; $CuSO_4.5H_2O$ (25.0% Cu), 0.9600; KI (76.9% I), 0.0668 g; Na_2SeO3 (44.7% Se), 0.0168. All ingredients were diluted with corn starch to 1 kg.
[c] Moisture, crude protein, digestible protein and crude lipid contents were measured value.
[d] n-3 and n-6 contents were calculated according to NRC (2011).

During the experiment, water temperature was averaged at 28 ± 2 °C, and pH value was maintained at 7.0 ± 0.2. The dissolved oxygen not less than 6.0 mg L^{-1} according to our laboratory study [43]. The experimental units were under natural light and dark cycle as described by Wen et al. [44].

2.3. Challenge trial

After the growth trial, a challenge trial was conducted to study the effects of dietary protein levels on the immune responses in the gill of grass carp. *F. columnare* was kindly provided by the College of Veterinary Medicine, Sichuan Agricultural University, China, and was used to cause gill disease in fish [45]. Fifteen fish with similar body weights were obtained from each treatment group as described by Shoemaker et al. [45] and acclimatized to the experimental conditions for 5 days according to Arias et al. [46]. At the end of the acclimatization period, fish were challenged by immersion exposure to 1.0×10^8 colony-forming units (cfu) ml^{-1} of *F. columnare* for 3 h after which the fish were returned to each experimental cage to feed for 3 days. The infection dose was sufficient to activate the immune system, and consequently, an investigation was conducted of effluent on reactivity against a threatening disease, according to our preliminary study data (unpublished data). During the 3 days challenge trial, fish were fed the same diets as in the feeding trial four times each day. Experimental conditions were identical to those in the growth trial.

2.4. Sample collection

At the end of the challenge trial, all fish from each treatment were anaesthetized in a benzocaine bath as described by Geraylou et al. [47]. Gill rot is the common gill disease of fish [3]. To investigate the effects of diets containing graded levels of protein on the resistance of fish against gill rot, a scoring system was designed to evaluate the severity of fish gill rot that was similar to Taylor et al. [3]. When sacrificed, fish gills were quickly removed, frozen in liquid nitrogen, and stored at −80 °C for later analysis as described by Chen et al. [7].

2.5. Biochemical analysis

The gill samples were homogenized in 10 vol (w v^{-1}) of ice-cold physiological saline and centrifuged at 6000 g at 4 °C for 20 min, and the supernatants were stored until used as described by Li et al. [48]. The gill lysozyme (LA) and acid phosphatase (ACP) activities, and complement 3 (C3) and complement 4 (C4) contents were assayed according to Zhao et al. [49]. The contents of reactive oxygen species (ROS), malondialdehyde (MDA), protein carbonyl (PC) and glutathione (GSH) in the gills were assayed as described by Feng et al. [50]. The activities of catalase (CAT) and glutathione peroxidase (GPx) in the gills were determined according to Chen et al. [7]. The total superoxide dismutase (SOD) and copper, zinc superoxide dismutase (CuZnSOD) activities in the gills were determined as described by Lu et al. [51]. The manganese superoxide dismutase (MnSOD) activity in the gills was calculated by deducting CuZnSOD from total SOD. The glutathione-*S*-transferases (GST) and glutathione reductase (GR) activities in the gills were measured according to Shi et al. [8].

2.6. Real-time polymerase chain reaction (PCR) analysis

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were similar to those previously described in another study conducted in our laboratory [52]. The total RNA was extracted from the gill samples using RNAiso Plus kit (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's instructions followed by DNAse I treatment. RNA quality and quantity were assessed using agarose gel (1%) electrophoresis and

spectrophotometric (A260: 280 nm ratio) analysis, respectively. Subsequently, RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa) according to the manufacturer's instructions. For quantitative real-time PCR, specific primers were designed according to the sequences cloned in our laboratory and the published sequences of grass carp (Table 2). According to the results of our preliminary experiment concerning the evaluation of internal control genes (data not shown), β-actin was used as a reference gene to normalize cDNA loading. The target and housekeeping gene amplification efficiency were calculated according to the specific gene standard curves generated from 10-fold serial dilutions. The $2^{-\Delta\Delta CT}$ method was used to calculate the expression results after verifying that the primers amplified with an efficiency of approximately 100% as described by Livak and Schmittgen [53].

2.7. Calculations and statistical analysis

The results were presented as the mean ± standard deviation (SD). All data were subjected to a one-way analysis of variance (ANOVA) by Duncan's multiple range tests to determine significant differences among the treatments at $P < 0.05$ with SPSS 18.0 (SPSS Inc., Chicago, IL, USA) according to Jiang et al. [54].

3. Results

3.1. Gill rot morbidity of fish after infection with F. columnare

Gill rot morbidity (Fig. 1) after infection with *F. columnare* gradually decreased with the increase in dietary protein levels up to 257.5 g digestible protein kg^{-1} diet and then gradually increased. After infection of grass carp with *F. columnare*, compared with low and high levels of protein, at the optimal level of dietary protein, the gill rot symptoms were clearly alleviated (Fig. 2).

3.2. Gill immune parameters

The effects of dietary protein levels on the activities of LA and ACP and the contents of C3 and C4 in the gills of grass carp are presented in Table 3, and these parameters all gradually increased with the increase in dietary protein levels up to 176.7, 257.5, 257.5 and 257.5 g digestible protein kg^{-1} diet, respectively, and then all gradually decreased. As shown in Fig. 3A, based on the quadratic regression analysis of ACP, the optimal level of dietary protein for grass carp was estimated to be 257.76 g digestible protein kg^{-1} diet.

3.3. Relative mRNA levels of antimicrobial peptides, cytokines and related signalling molecules in the gills

The effects of dietary protein levels on immune-related indices in the gills of grass carp are presented in Figs. 4–6. The mRNA levels for liver-expressed antimicrobial peptide 2A (LEAP-2A), LEAP-2B, Hepcidin and β-defensin in the gills of grass carp were gradually up-regulated with increasing dietary protein levels up to 257.5, 217.2, 257.5 and 257.5 g digestible protein kg^{-1} diet, respectively, and then all gradually down-regulated.

The mRNA levels for IL-1β, IL-12p40, interferon γ2 (IFN-γ2), TNF-α, NF-κB P65, NF-κB P52, IκB kinase α (IKKα), IKKβ, eIF4E-binding proteins 1 (4E-BP1) and 4E-BP2 in the gills of grass carp were gradually down-regulated with increasing levels of dietary protein up to 257.5, 257.5, 257.5, 257.5, 292.2, 217.2, 257.5, 257.5, 257.5 and 217.2 g digestible protein kg^{-1} diet, respectively; above these levels, all were gradually up-regulated. The mRNA level of IL-8 in the gills of grass carp reached the lowest level at 217.2 g digestible protein kg^{-1} diet ($P < 0.05$). For IKKγ in the gills of grass carp, the mRNA level was gradually down-regulated with increasing dietary protein levels up to 176.7 g digestible protein kg^{-1} diet; above this value, the level plateaued ($P > 0.05$).

The mRNA levels of IL-6, IL-11, transforming growth factor β2 (TGF-β2), inhibitor of κBα (IκBα), TOR and ribosomal protein S6 kinases 1 (S6K1) in the gills of grass carp were gradually up-regulated with increasing dietary protein levels up to 257.5, 257.5, 217.2, 257.5, 217.2 and 217.2 g digestible protein kg^{-1} diet, respectively; the levels were then all gradually down-regulated. Fish fed 257.5 g digestible protein kg^{-1} diet showed the maximum mRNA level for TGF-β1 in the gills. However, the mRNA level of IL-10 was not significantly different in the gills at different levels of dietary protein ($P > 0.05$).

3.4. Antioxidant-related parameters, Nrf2, Keap1a and Keap1b relative mRNA levels in the gills

The effects of dietary protein levels on the gill antioxidant-related parameters of grass carp are shown in Table 4. The contents of ROS and PC in the gills of grass carp both gradually decreased with the increase in dietary protein levels up to 257.5 g digestible protein kg^{-1} diet above which the contents gradually increased. The MDA content in the gills of grass carp decreased significantly with increasing dietary protein levels up to 257.5 g digestible protein kg^{-1} diet ($P < 0.05$) above which the content plateaued ($P > 0.05$). The activities of CAT, GPx, GST and GR in the gills of grass carp were gradually increased with increasing dietary protein levels up to 217.2, 217.2, 257.5 and 217.2 g digestible protein kg^{-1} diet, respectively; above these levels, all were gradually decreased. The GSH content in the gills of grass carp was gradually increased with increasing dietary protein levels up to 217.2 g digestible protein kg^{-1} diet above which the content plateaued ($P > 0.05$). However, the activity of CuZnSOD was not significantly different in the gills with different dietary protein levels ($P > 0.05$). The activity of MnSOD in the gills of grass carp was gradually decreased with increasing dietary protein levels up to 292.2 g digestible protein kg^{-1} diet, and then gradually increased. As shown in Fig. 3B, based on the quadratic regression analysis of PC, the optimal dietary protein level for grass carp was estimated to be 260.69 g digestible protein kg^{-1} diet.

The effects of dietary protein levels on antioxidant enzymes and related signalling molecules relative mRNA levels in the gills of grass carp are presented in Fig. 7. The gene expression of MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTR, GSTO, GR and Nrf2 were gradually up-regulated with increasing dietary protein levels up to 257.5, 257.5, 257.5, 257.5, 257.5, 217.2, 257.5, 257.5, 257.5 and 257.5 g digestible protein kg^{-1} diet in the gills of grass carp, respectively, and then all gradually down-regulated. The CuZnSOD mRNA level in the gills of grass carp was gradually up-regulated with increasing dietary protein levels up to 176.7 g digestible protein kg^{-1} diet, and then plateaued ($P > 0.05$). The gene expression of Kelch-like-ECH-associated protein 1a (Keap1a) and Keap1b in the gills of grass carp were both gradually down-regulated with increasing dietary protein levels up to 257.5 and 217.2 g digestible protein kg^{-1} diet, respectively, and then gradually up-regulated.

3.5. Relative mRNA levels of apoptosis-related parameters in the gills

The effects of dietary protein levels on apoptosis and related signalling molecules relative mRNA levels in the gills of grass carp are presented in Fig. 8. The cysteinyl aspartic acid-protease 3 (caspase 3), caspase 8, caspase 9, apoptotic protease activating factor-1 (Apaf-1), Bcl-2 associated X protein (Bax), fatty acid synthetase ligand (FasL) and JNK mRNA levels in the gills of grass carp

Table 2
Real-time PCR primer sequences.[a]

Target gene	Primer sequence forward (5′→3′)	Primer sequence reverse (5′→3′)	Temperature (°C)	Accession number
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59.3	JQ246442.1
LEAP-2A	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59.3	FJ390414
LEAP-2B	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59.3	KT625603
β-defensin	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58.4	KT445868
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	60.4	JX657682
TNF-α	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC	58.4	HQ696609
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	57.1	JQ692172
IL-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	62.3	KC535507.1
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	60.3	JN663841
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	61.4	HQ388294
IL-11	GGTTCAAGTCTCTTCCAGCGAT	TGCGTGTTATTTTGTTCAGCCA	57.0	KT445870
IL-12p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55.4	KF944667.1
IL-12p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	59.0	KF944668.1
TGF-β1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	55.9	EU099588
TGF-β2	TACATTGACAGCAAGGTGGTG	TCTTGTTGGGGATGATGTAGTT	55.9	KM279716
NF-κB P52	TCAGTGTAACGACAACGGGAT	ATACTTCAGCCACACCTCTCTTAG	58.4	KM279720
NF-κB P65	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	62.3	KJ526214
IκBα	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA	62.3	KJ125069
IKKα	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA	60.3	KM279718
IKKβ	GTGGCGGTGGATTATTGG	GCACGGGTTGCCAGTTTG	60.3	KP125491
IKKγ	AGAGGCTCGTCATAGTGG	CTGTGATTGGCTTGCTTT	58.4	KM079079
TOR	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	61.4	JX854449
S6K1	TGGAGGAGGTAATGGACG	ACATAAAGCAGCCTGACG	54.0	EF373673
4E-BP1	GCTGGCTGAGTTTGTGGTTG	CGAGTCGTGCTAAAAAGGGTC	60.3	KT757305
4E-BP2	CACTTTATTCTCCACCACCCC	TTCATTGAGGATGTTCTTGCC	60.3	KT757306
occludin	TATCTGTATCACTACTGCGTCG	CATTCACCCAATCCTCCA	59.4	KF193855
ZO-1	CGGTGTCTTCGTAGTCGG	CAGTTGGTTTGGGTTTCAG	59.4	KJ000055
ZO-2	TACAGCGGGACTCTAAAATGG	TCACACGGTCGTTCTCAAAG	60.3	KM112095
claudin b	GAGGGAATCTGGATGAGC	ATGGCAATGATGGTGAGA	57.0	KF193860
claudin c	GAGGGAATCTGGATGAGC	CTGTTATGAAAGCGGCAC	59.4	KF193859
claudin 3	ATCACTCGGGACTTCTA	CAGCAAACCCAATGTAG	57.0	KF193858
claudin 7a	ACTTACCAGGGACTGTGGATGT	CACTATCATCAAAGCACGGGT	59.3	KT625604
claudin 7b	CTAACTGTGGTGGTGATGAC	AACAATGCTACAAAGGGCTG	59.3	KT445866
claudin 12	CCCTGAAGTGCCCACAA	GCGTATGTCACGGGAGAA	55.4	KF998571
claudin 15a	TGCTTTATTTCTTGGCTTTC	CTCGTACAGGGTTGAGGTG	59.0	KF193857
claudin 15b	AGTGTTCTAAGATAGGAGGGGAG	AGCCCTTCTCCGATTTCAT	62.3	KT757304
MLCK	GAAGGTCAGGGCATCTCA	GGGTCGGGCTTATCTACT	53.0	KM279719
FasL	AGGAAATGCCCGCACAAATG	AACCGCTTTCATTGACCTGGAG	61.4	KT445873
p38 MAPK	TGGGAGCAGACCTCAACAAT	TACCATCGGGTGGCAACATA	60.4	KM112098
JNK	ACAGCGTAGATGTGGGTGATT	GCTCAAGGTTGTGGTCATACG	62.3	KT757312
Bcl-2	AGGAAAATGGAGGTTGGGAT	CTGAGCAAAAAAGGCGATG	60.3	JQ713862.1
Mcl-1	TGGAAAGTCTCGTGGTAAAGCA	ATCGCTGAAGATTTCTGTTGCC	58.4	KT757307
Bax	CATCTATGAGCGGGTTCGTC	TTTATGGCTGGGGTCACACA	60.3	JQ793788.1
Apaf-1	AAGTTCTGGAGCCTGGACAC	AACTCAAGACCCCACAGCAC	61.4	KM279717
IAP	CACAATCCTGGTATGCGTCG	GGGTAATGCCTCTGGTGCTC	58.4	FJ593503.1
caspase 3	GCTGTGCTTCATTTGTTTG	TCTGAGATGTTATGGCTGTC	55.9	JQ793789
caspase 8	ATCTGGTTGAAATCCGTGAA	TCCATCTGATGCCCATACAC	59.0	KM016991
caspase 9	CTGTGGCGGAGGTGAGAA	GTGCTGGAGGACATGGGAAT	59.0	JQ793787
CuZnSOD	CGCACTTCAACCCTTACA	ACTTTCCTCATTGCCTCC	61.5	GU901214
MnSOD	ACGACCCAAGTCTCCCTA	ACCCTGTGGTTCTCCTCC	60.4	GU218534
CAT	GAAGTTCTACACCGATGAGG	CCAGAAATCCCAAACCAT	58.7	FJ560431
GPx1a	GGGCTGGTTATTCTGGGC	AGGCGATGTCATTCCTGTTC	61.5	EU828796
GPx1b	TTTTGTCCTTGAAGTATGTCCGTC	GGGTCGTTCATAAAGGGCATT	60.3	KT757315
GPx4a	TACGCTGAGAGAGGTTTACACAT	CTTTTCCATTGGGTTGTTCC	60.4	KU255598
GPx4b	CTGGAGAAATACAGGGGTTACG	CTCCTGCTTTCCGAACTGGT	60.3	KU255599
GSTR	TCTCAAGGAACCCGTCTG	CCAAGTATCCGTCCCACA	58.4	EU107283
GSTO	GGTGCTCAATGCCAAGGGAA	CTCAAACGGGTCGGATGGAA	58.4	KT757314
GR	GTGTCCAACTTCTCCTGTG	ACTCTGGGGTCCAAAACG	59.4	JX854448
Nrf2	CTGGACGAGGAGACTGGA	ATCTGTGGTAGGTGGAAC	62.5	KF733814
Keap1a	TTCCACGCCCTCCTCAA	TGTACCCTCCCGCTATG	63.0	KF811013
Keap1b	TCTGCTGTATGCGGTGGGC	CTCCTCCATTCATCTTTCTCG	57.9	KJ729125
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	61.4	M25013

[a] LEAP-2, liver expressed antimicrobial peptide 2; IFN-γ2, interferon γ2; TNF-α, tumor necrosis factor α; IL, interleukin; TGF-β, transforming growth factor β; NF-κB, nuclear factor kappa B; IκBα, inhibitor of κBα; IKK, IκB kinase; TOR, target of rapamycin; S6K1, ribosomal protein S6 kinases 1; 4E-BP, eIF4E-binding proteins; ZO, zonula occludens; MLCK, myosin light chain kinase; FasL, fatty acid synthetase ligand; p38 MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal protein kinase; Bcl-2, B-cell lymphoma protein-2; Mcl-1, myeloid cell leukemia-1; Bax, Bcl-2 associated X protein; Apaf-1, apoptotic protease activating factor-1; IAP, inhibitor of apoptosis proteins; caspase, cysteinyl aspartic acid-protease; CuZnSOD, copper, zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase; Nrf2, NF-E2-related factor 2; Keap1, Kelch-like-ECH-associated protein 1.

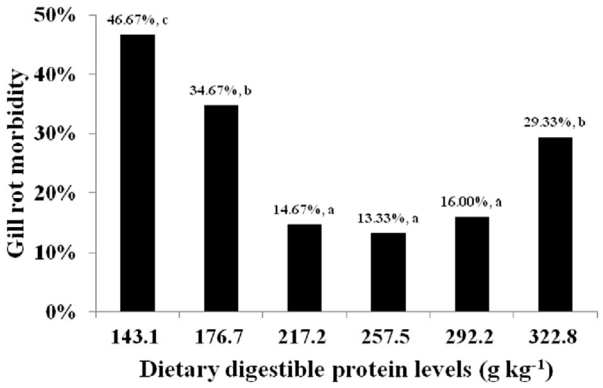

Fig. 1. The gill rot morbidity of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein after infection with *Flavobacterium columnare* for 3 days.

dietary protein levels up to 257.5, 257.5 and 217.2 g digestible protein kg^{-1} diet, and then all gradually down-regulated.

3.6. Relative mRNA levels of TJs, MLCK and p38 MAPK in the gills

The effects of dietary protein levels on intercellular TJs, MLCK and p38 mitogen-activated protein kinase (p38 MAPK) relative mRNA levels in the gills of grass carp are presented in Fig. 9. Fish fed 292.2, 257.5, 257.5, 217.2, 257.5, 257.5, 257.5, 257.5, 257.5 and 257.5 g digestible protein kg^{-1} diet showed the maximum mRNA levels of occludin, ZO-1, ZO-2, claudin c, claudin 3, claudin 7a, claudin 7b, claudin 12, claudin 15a and claudin 15b in the gills, respectively, and then all gradually down-regulated. The claudin b mRNA level in the gills of grass carp was significantly up-regulated with increasing dietary protein levels up to 176.7 g digestible protein kg^{-1} diet, and then plateaued ($P > 0.05$). The MLCK and p38 MAPK mRNA levels in the gills of grass carp were both down-regulated with increasing dietary protein levels up to 257.5 g digestible protein kg^{-1} diet, and then gradually up-regulated.

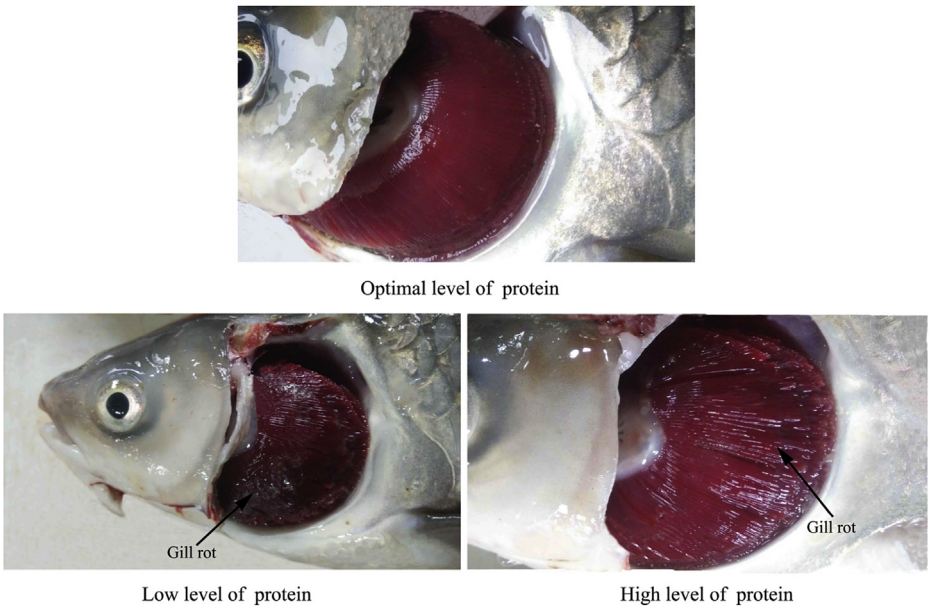

Fig. 2. Compared with low and high levels of protein, optimal dietary protein level obviously alleviated gill rot symptom after infection with *F. columnare* in grass carp.

Table 3
Lysozyme (LA, U mg^{-1} protein), acid phosphatase (ACP, U mg^{-1} protein) activities, complement 3 (C3, mg g^{-1} protein) and complement 4 (C4, mg g^{-1} protein) contents in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks.

	Dietary protein levels (dietary digestible protein levels) (g kg^{-1})					
	169.2 (143.1)	204.7 (176.7)	244.3 (217.2)	283.2 (257.5)	323.2 (292.2)	366.3 (322.8)
LA	60.57 ± 5.88^{a}	133.91 ± 13.00^{b}	133.59 ± 12.32^{b}	123.82 ± 10.20^{b}	123.52 ± 3.23^{b}	122.04 ± 10.28^{b}
ACP	114.02 ± 6.98^{a}	183.85 ± 13.87^{b}	204.99 ± 16.06^{cd}	237.27 ± 10.48^{e}	218.51 ± 14.14^{de}	192.75 ± 26.81^{bc}
C3	13.13 ± 0.83^{a}	16.11 ± 1.19^{b}	18.31 ± 1.18^{c}	19.38 ± 0.89^{c}	18.75 ± 1.29^{c}	18.43 ± 1.13^{c}
C4	1.45 ± 0.11^{a}	1.67 ± 0.15^{b}	2.31 ± 0.21^{d}	2.49 ± 0.22^{d}	1.89 ± 0.18^{c}	1.69 ± 0.13^{bc}
Regression						
$Y_{C3} = 0.0693x + 3.4431$; $Y_{Max} = 18.7170$					$R^2 = 0.9802$	$P = 0.090$
$Y_{C4} = -1.0092 \times 10^{-4}x^2 + 0.0488x - 3.5749$					$R^2 = 0.8388$	$P = 0.065$

[1] Values are means ± SD (n = 6), and different superscripts in the same row are significantly different ($P < 0.05$).

were gradually down-regulated with increasing dietary protein levels up to 257.5, 217.2, 257.5, 257.5, 217.2, 217.2 and 257.5 g digestible protein kg^{-1} diet, respectively, and then all gradually up-regulated.

The B-cell lymphoma protein-2 (Bcl-2), inhibitor of apoptosis proteins (IAP) and myeloid cell leukemia-1 (Mcl-1) mRNA levels in the gills of grass carp were gradually up-regulated with increasing

4. Discussion

This study used the identical growth trial as that in our previous study [4] and was a part of a larger research effort to determine the

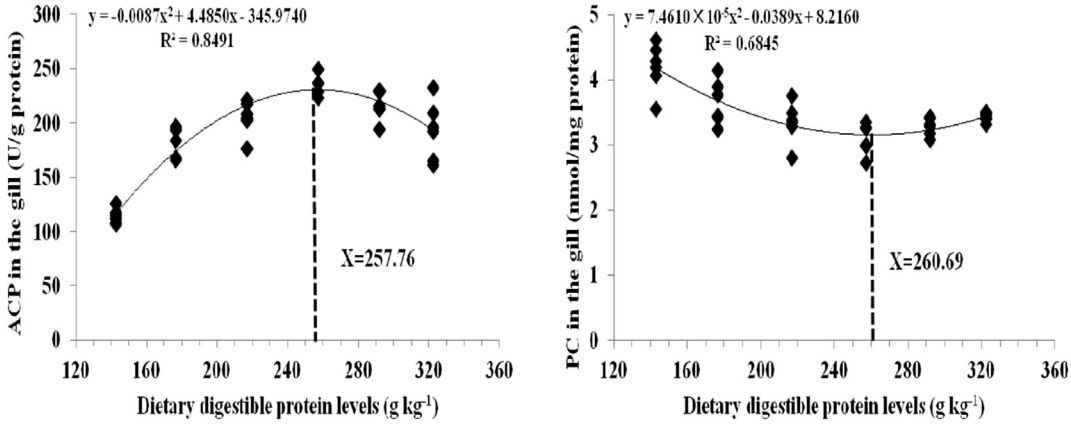

Fig. 3. Quadratic regression analysis of ACP activity (A) and PC content (B) in the gill of grass carp fed diets containing graded levels of protein for 8 weeks. ACP, acid phosphatase; PC, protein carbonyl.

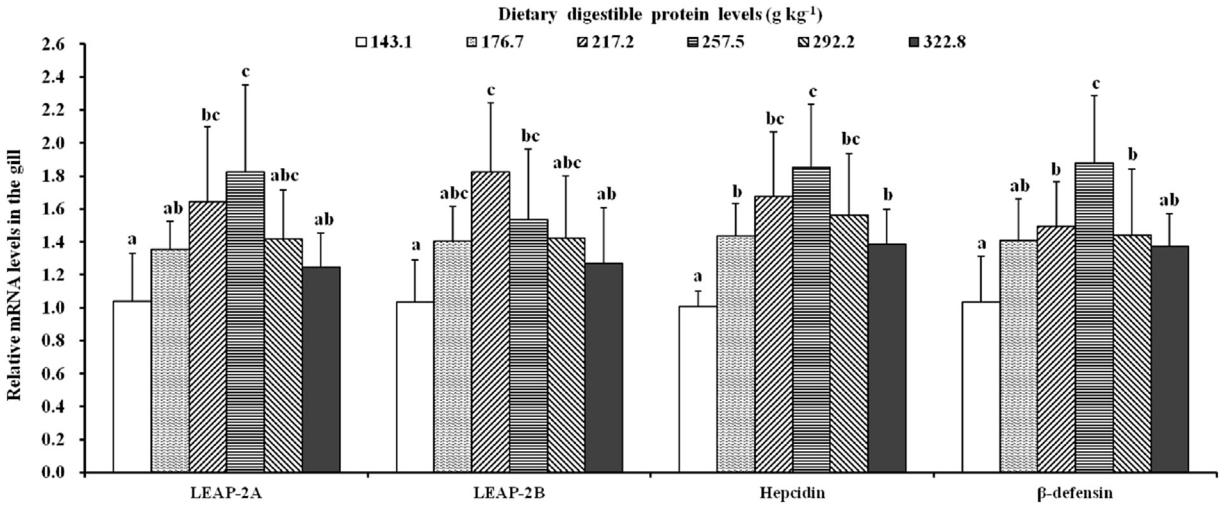

Fig. 4. Relative expression of LEAP-2A, LEAP-2B, Hepcidin and β-defensin in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). LEAP-2, liver-expressed antimicrobial peptide 2.

effects of dietary protein levels on the growth, immune function and physical barrier function in fish. In a previous study, at the optimal level of dietary protein, the growth of grass carp increased [4]. In fish, in general, growth is dependent on gill health [2]. Thus, in the current study, first, the effects of dietary protein levels on gill health in fish were investigated.

4.1. Optimal level of dietary protein improved fish resistance to disease

Based on our understanding, fish gill health is highly correlated with the resistance to disease [55]. Moreover, *F. columnare*, the etiological agent of columnaris disease, is distributed world-wide in aquatic environments and is an established pathogen of fresh-water fish [56]. *F. columnare* induces marked pathologic changes in numerous ectopic tissues, and the adhesion of *F. columnare* to the gill results in pronounced erosion and necrosis of external tissues [57,58]. Sun et al. [59] found that *F. columnare* infection triggers the immune events, which were related to the immune function in the gill of channel catfish. Thus, after the feeding trial, to investigate the resistance of fish against gill rot, we infected the fish with *F. columnare* and evaluated the gill rot morbidity. In this study, we observed that low and high levels of protein caused 46.67% and 29.33% gill rot morbidity, respectively, whereas at an optimal level of dietary protein, the gill rot morbidity decreased to 13.33% in grass carp after infection with *F. columnare*, suggesting that optimal level of dietary protein improved fish resistance against gill rot. Based on the quadratic regression analysis of protecting fish against gill rot morbidity with different levels of dietary protein ($Y_{\text{gill rot morbidity}} = 0.0030x^2 - 1.5224x + 204.9639$, $R^2 = 0.9621$, $P < 0.01$), the optimal dietary protein level for grass carp was estimated to be 253.73 g digestible protein kg^{-1} diet. Fish gill health is also closely correlated with the immune function [5] and physical barrier function [6]. Therefore, we next investigated the effects of dietary protein levels on the gill immune function and physical barrier function of grass carp.

4.2. Optimal level of dietary protein enhanced immune function in the gills of fish

4.2.1. Optimal level of dietary protein increased antibacterial compounds in the gills of fish

The immune function of fish primarily depends on the immune response, which is closely correlated with antibacterial compounds

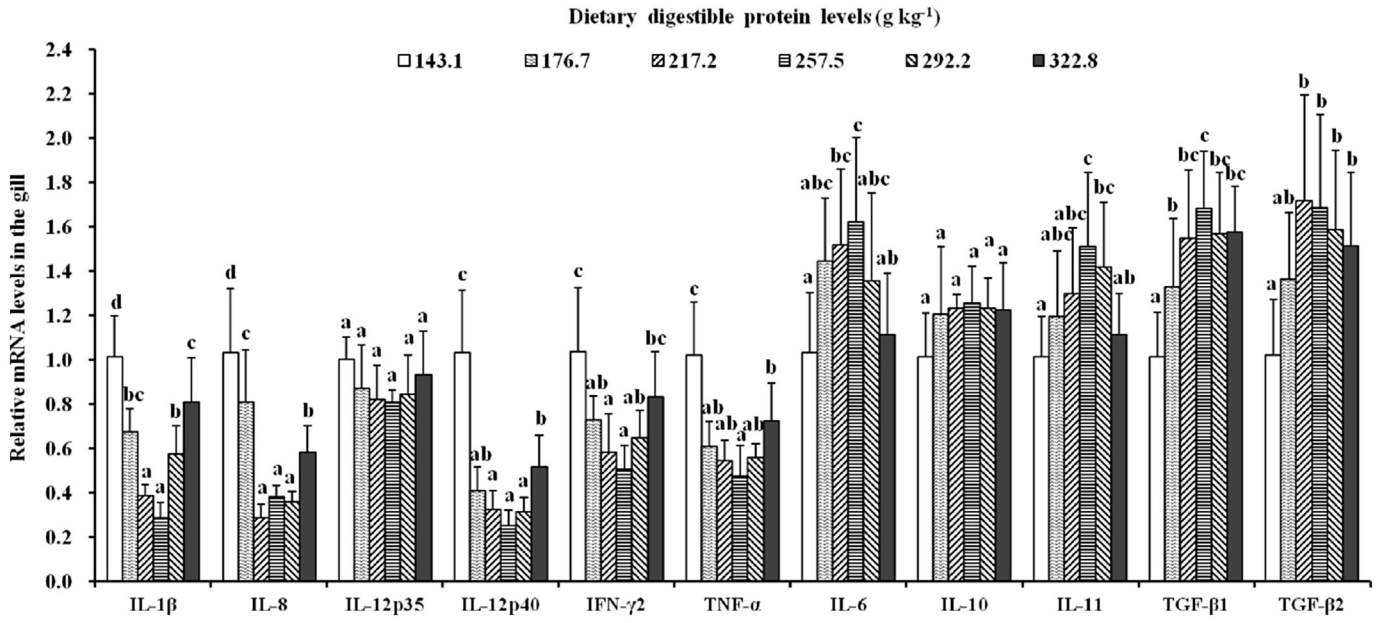

Fig. 5. Relative expression of inflammatory cytokines IL-1β, IL-8, IL-12p35, IL-12p40, IFN-γ2, TNF-α, IL-6, IL-10, IL-11, TGF-β1 and TGF-β2 in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). IL, interleukin; IFN-γ2, interferon γ2; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor β.

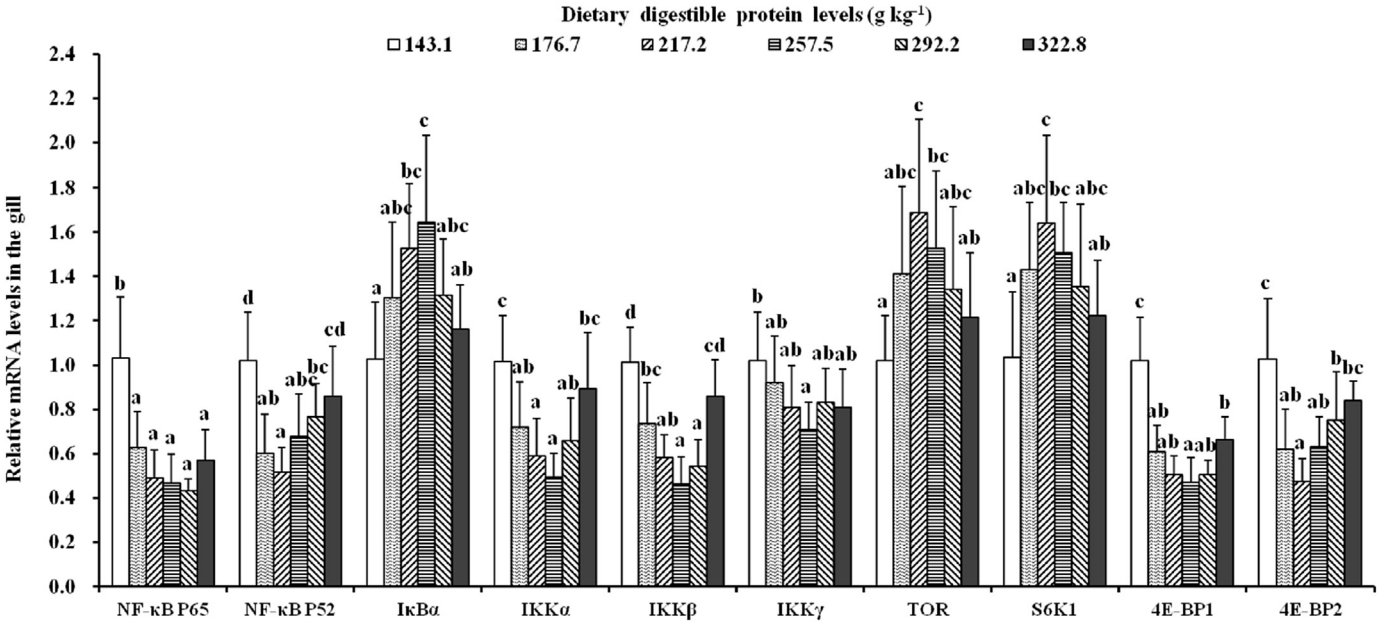

Fig. 6. Relative expression of NF-κB P65, NF-κB P52, IκBα, IKKα, IKKβ, IKKγ, TOR, S6K1, 4E-BP1 and 4E-BP2 in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). NF-κB, nuclear factor kappa B; IκBα, inhibitor of κBα; IKK, IκB kinase; TOR, target of rapamycin; S6K1, ribosomal protein S6 kinases 1; 4E-BP, eIF4E-binding proteins.

such as LA, ACP, complements and antimicrobial peptides [10,60]. According to Chen et al. [7], the gill immune function of grass carp is enhanced with an increase in LA and ACP activities and C3 content and the up-regulation of LEAP-2 and Hepcidin mRNA levels. In this study, compared with low or high levels of protein, at an optimal level of dietary protein, the activities of LA and ACP and contents of C3 and C4 increased, and the mRNA levels of LEAP-2A, LEAP-2B, Hepcidin and β-defensin were up-regulated in the gills of grass carp. These data indicated that at an optimal level of dietary protein the gill immune function of fish improved. According to a report, the immune function in fish is closely correlated with the inflammation response, which is primarily mediated by cytokines [61]. Hence, we next investigated the relationship between dietary protein levels and cytokines in the gills of grass carp.

4.2.2. Optimal level of dietary protein attenuated inflammatory response partly through NF-κB and TOR signalling pathways in the gills of fish

Based on our understanding, inflammatory cytokines in fish are classified as pro-inflammatory cytokines (such as IL-1β, IL-8 and

Table 4
MDA (nmol mg^{-1} protein), PC (nmol mg^{-1} protein) and ROS (% DCF florescence) contents, and activities of CuZnSOD (U mg^{-1} protein), MnSOD (U mg^{-1} protein), CAT (U mg^{-1} protein), GPx (U mg^{-1} protein), GST (U mg^{-1} protein) and GR (U mg^{-1} protein), and GSH (mg g^{-1} protein) content in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks.[a]

	Dietary protein levels (dietary digestible protein levels) (g kg^{-1})					
	169.2 (143.1)	204.7 (176.7)	244.3 (217.2)	283.2 (257.5)	323.2 (292.2)	366.3 (322.8)
ROS	100.00 ± 8.20^{c}	98.05 ± 6.28^{c}	54.30 ± 4.50^{b}	41.52 ± 3.78^{a}	42.05 ± 2.82^{a}	39.56 ± 3.29^{a}
MDA	2.29 ± 0.15^{d}	2.11 ± 0.12^{c}	1.85 ± 0.17^{b}	1.45 ± 0.10^{a}	1.37 ± 0.13^{a}	1.30 ± 0.07^{a}
PC	4.19 ± 0.37^{c}	3.65 ± 0.34^{b}	3.34 ± 0.31^{ab}	3.09 ± 0.24^{a}	3.28 ± 0.14^{a}	3.42 ± 0.07^{ab}
CuZnSOD	3.64 ± 0.66^{a}	3.81 ± 0.51^{a}	3.74 ± 0.64^{a}	3.27 ± 0.58^{a}	3.80 ± 0.59^{a}	3.58 ± 0.44^{a}
MnSOD	3.63 ± 1.20^{d}	3.41 ± 1.11^{cd}	2.96 ± 0.57^{bcd}	2.46 ± 0.52^{abc}	1.60 ± 0.55^{a}	2.19 ± 0.83^{ab}
CAT	0.63 ± 0.05^{bc}	0.63 ± 0.06^{bc}	0.68 ± 0.05^{c}	0.58 ± 0.05^{ab}	0.56 ± 0.05^{a}	0.56 ± 0.05^{a}
GPx	118.46 ± 5.29^{a}	125.74 ± 8.46^{ab}	131.50 ± 7.90^{b}	125.27 ± 6.57^{ab}	120.86 ± 9.20^{ab}	117.21 ± 11.46^{a}
GST	63.17 ± 2.79^{a}	70.79 ± 5.59^{b}	80.08 ± 6.05^{c}	81.05 ± 5.54^{c}	75.64 ± 7.01^{bc}	68.79 ± 6.01^{ab}
GR	19.64 ± 1.75^{a}	21.37 ± 2.03^{a}	24.24 ± 2.11^{b}	23.78 ± 2.32^{b}	20.89 ± 1.35^{a}	20.02 ± 1.32^{a}
GSH	1.17 ± 0.11^{a}	1.28 ± 0.11^{a}	1.45 ± 0.08^{b}	1.47 ± 0.11^{b}	1.42 ± 0.09^{b}	1.47 ± 0.11^{b}
Regression						
$Y_{ROS} = -0.5762x + 187.9058$; $Y_{Min} = 41.0435$					$R^2 = 0.9080$	$P < 0.05$
$Y_{MDA} = -0.0073x + 3.3720$; $Y_{Min} = 1.3753$					$R^2 = 0.9821$	$P < 0.01$
$Y_{MnSOD} = -0.0104x + 5.1759$; $Y_{Max} = 2.0807$					$R^2 = 0.9860$	$P < 0.01$
$Y_{GPx} = -0.0014x^2 + 0.6268x + 58.0601$					$R^2 = 0.8695$	$P < 0.05$
$Y_{GST} = -0.0018x^2 + 0.8967x - 28.2008$					$R^2 = 0.9781$	$P < 0.01$
$Y_{GR} = -5.2240 \times 10^{-4}x^2 + 0.2439x - 4.7614$					$R^2 = 0.8752$	$P < 0.05$

[a] Values are means ± SD (n = 6), and different superscripts in the same row are significantly different ($P < 0.05$). ROS, reactive oxygen species; MDA, malondialdehyde; PC, protein carbonyl; CuZnSOD, copper, zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase and GSH, glutathione.

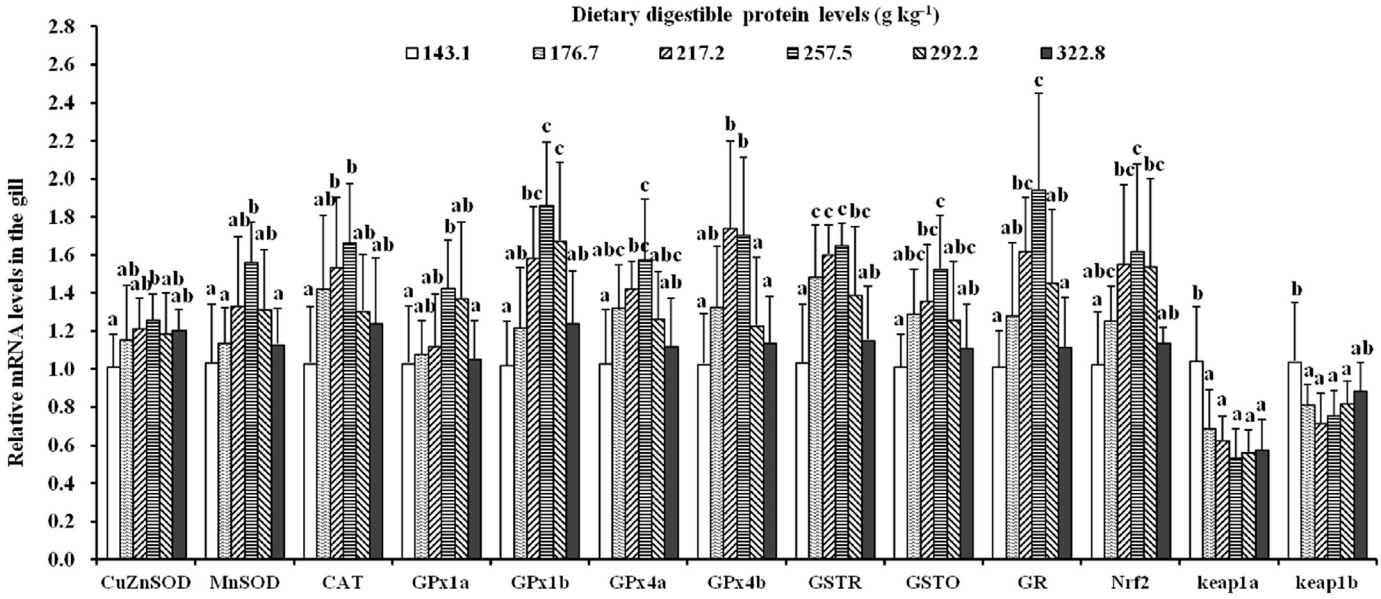

Fig. 7. Relative expression of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTR, GSTO, GR, Nrf2, Keap1a and Keap1b in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). CuZnSOD, copper, zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase; Nrf2, NF-E2-related factor 2; Keap1, Kelch-like-ECH-associated protein 1.

TNF-α) and anti-inflammatory cytokines (such as IL-6, IL-10 and TGF-β1), and down-regulated pro-inflammatory cytokines IL-1β, IL-8 and TNF-α mRNA levels and up-regulated anti-inflammatory cytokines IL-6, IL-10 and TGF-β1 mRNA levels could attenuate inflammation [7,62,63]. In the present study, compared with low or high levels of protein, the optimal levels of dietary protein led to the down-regulation of the pro-inflammatory cytokines IL-1β, IL-8, IFN-γ2 and TNF-α mRNA levels and the up-regulation of the anti-inflammatory cytokines IL-6, IL-11, TGF-β1 and TGF-β2 mRNA levels in the gills of grass carp. These results suggested that optimal level of dietary protein could attenuate gill inflammation in fish. Notably, compared with low or high levels of protein, the mRNA level were down-regulated for the pro-inflammatory cytokines IL-12p40 at the optimal level of dietary protein, but we found no significant effect on the mRNA level of IL-12p35. In this case, for this study, at different levels of dietary protein, IL-1β might regulate the mRNA level of IL-12p40 (rather than IL-12p35). As reported for Atlantic salmon (*Salmo salar*), IL-1β up-regulates the mRNA level of IL-12p40 but has no effect on the mRNA level of IL-12p35 [64]. In this study, at the optimal level of dietary protein, the IL-1β mRNA level was down-regulated in the gills of grass carp. Correlation analysis showed that the mRNA level of IL-12p40 was positively correlated with the mRNA level of IL-1β in the gills of grass carp (Table 5). According to the above data, we presumed that at

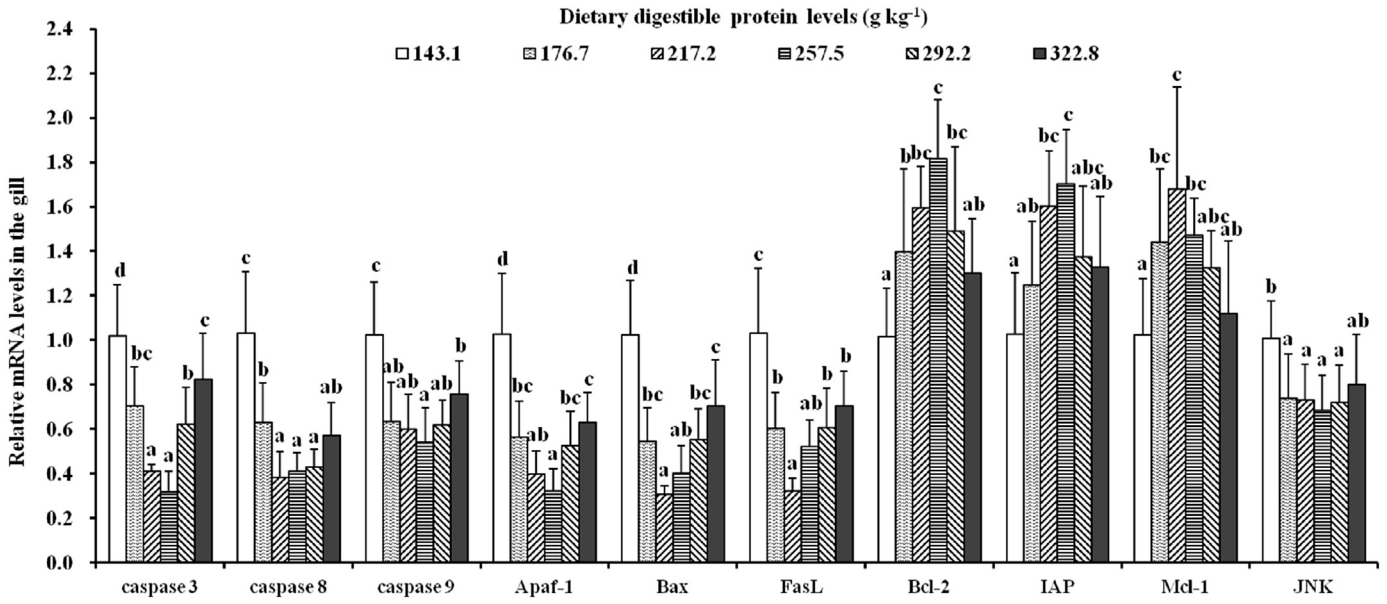

Fig. 8. Relative expression of caspase 3, caspase 8, caspase 9, Apaf-1, Bax, FasL, Bcl-2, IAP, Mcl-1 and JNK in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). Caspase, cysteinyl aspartic acid-protease; Apaf-1, apoptotic protease activating factor-1; Bax, Bcl-2 associated X protein; FasL, fatty acid synthetase ligand; Bcl-2, B-cell lymphoma protein-2; IAP, inhibitor of apoptosis proteins; Mcl-1, myeloid cell leukemia-1; JNK, c-Jun N-terminal protein kinase.

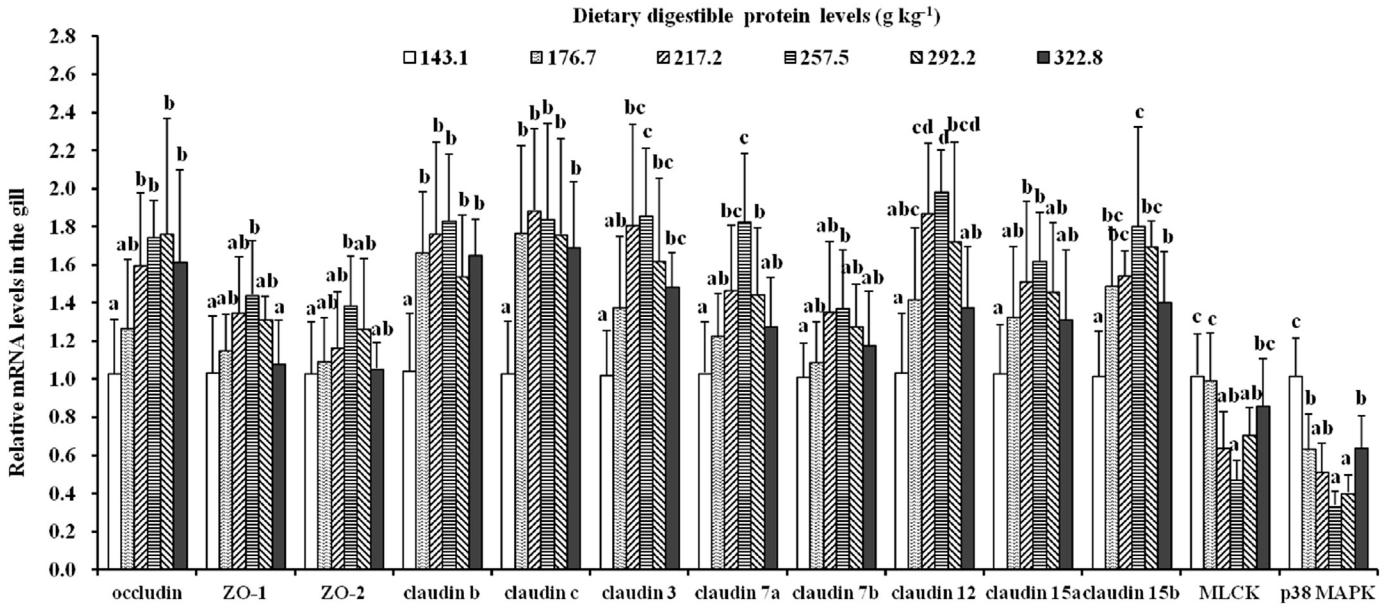

Fig. 9. Relative expression of occludin, ZO-1, ZO-2, claudin b, claudin c, claudin 3, claudin 7a, claudin 7b, claudin 12, claudin 15a, claudin 15b, MLCK and p38 MAPK in the gill of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of protein for 8 weeks. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). ZO, zonula occludens; MLCK, myosin light chain kinase; p38 MAPK, p38 mitogen-activated protein kinase.

different levels of dietary protein might regulate IL-12p40 (rather than IL-12p35) through IL-1β in fish gills, although further investigation is required. Notably, we also found that different levels of dietary protein did not alter gene expression of IL-10, with this phenomenon possibly related to IL-12p35. According to a report, the down-regulation of IL-12p35 expression increases IL-10 production in humans [65]. In this study, we found that different dietary protein levels had no significant effect on the IL-12p35 in the gills of grass carp. Hence, the absence of change in mRNA level of IL-10 in the gills of grass carp at different levels of dietary protein might be partly attributed to the IL-12p35 in fish gills. However, to test this hypothesis, further investigations are required.

As reported, the signalling molecule NF-κB plays a critical role in mediating gene expression of inflammatory cytokines in humans [66]. Min et al. [67] found that the inhibition of NF-κB activation decreases levels of the pro-inflammatory cytokines IL-1β, IL-8 and TNF-α in human mast cell lines. It was reported that NF-κB P65 and NF-κB P52 are the subunits of NF-κB in fish [68]. In the present study, compared with low or high levels of protein, we found that at the optimal levels of dietary protein down-regulated NF-κB P65

Table 5
Correlation coefficient of parameters in the gill of grass carp.

Independent parameters	Dependent parameters	Correlation coefficients	*P*
IL-1β	IL-12p40	+0.878	<0.05
NF-κB P65	IL-1β	+0.818	<0.05
	IL-8	+0.913	<0.05
	TNF-α	+0.946	<0.01
	IFN-γ2	+0.894	<0.05
	IL-12p40	+0.973	<0.01
NF-κB P52	IL-1β	+0.803	=0.055
	TNF-α	+0.836	<0.05
	IFN-γ2	+0.817	<0.05
	IL-12p40	+0.812	<0.05
IκBα	NF-κB P65	−0.745	=0.089
	NF-κB P52	−0.794	=0.059
	IKKα	−0.971	<0.01
	IKKβ	−0.915	<0.05
	IKKγ	−0.810	=0.051
TOR	IL-6	+0.913	<0.05
	IL-11	+0.699	=0.122
	TGF-β1	+0.670	=0.145
	TGF-β2	+0.843	<0.05
GPx4a mRNA level	GPx activity	+0.769	=0.074
GPx4b mRNA level	GPx activity	+0.879	<0.05
GSTR mRNA level	GST activity	+0.922	<0.01
GSTO mRNA level	GST activity	+0.926	<0.01
GR mRNA level	GR activity	+0.893	<0.05
Nrf2	CAT	+0.835	<0.05
	GPx1a	+0.817	<0.05
	GPx1b	+0.967	<0.01
	GPx4a	+0.877	<0.05
	GPx4b	+0.822	<0.05
	GSTR	+0.880	<0.05
	GSTO	+0.888	<0.05
	GR	+0.931	<0.01
Keap1a	Nrf2	−0.713	=0.112
Keap1b	Nrf2	−0.871	<0.05
FasL	caspase 8	+0.921	<0.01
JNK	FasL	+0.876	<0.05
caspase 9	caspase 3	+0.899	<0.05
Apaf-1	caspase 3	+0.944	<0.01
Bax	caspase 3	+0.940	<0.01
Bcl-2	caspase 3	−0.982	<0.01
Mcl-1	caspase 3	−0.877	<0.05
IAP	caspase 3	−0.965	<0.01
MLCK	occludin	−0.836	<0.05
	ZO-1	−0.936	<0.01
	ZO-2	−0.897	<0.05
	claudin b	−0.677	=0.140
	claudin 3	−0.923	<0.01
	claudin7a	−0.961	<0.01
	claudin 7b	−0.968	<0.01
	claudin 12	−0.938	<0.01
	claudin 15a	−0.903	<0.05
	claudin 15b	−0.818	<0.05
p38 MAPK	MLCK	+0.844	<0.05

and NF-κB P52 mRNA levels in the gills of grass carp. Correlation analysis indicated that the mRNA levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-8, IFN-γ2 and IL-12p40 were positively correlated with the mRNA levels of NF-κB P65 and NF-κB P52 in the gills of grass carp (Table 5). The nuclear translocation of NF-κB may be inhibited by IκBα in humans [69], and Heissmeyer et al. [70] reported that the IKK complex (including IKKα, IKKβ and IKKγ) catalyses IκBα degradation in 293 cells. In the present study, compared with low or high levels of protein, we found that the optimal levels of dietary protein led to the down-regulation of IKKα, IKKβ and IKKγ mRNA levels and the up-regulation of IκBα mRNA level in the gills of grass carp. Correlation analysis indicated that the mRNA levels of NF-κB P65 and NF-κB P52 were negatively correlated with that of IκBα and that the mRNA level of IκBα was negatively correlated with the mRNA levels of IKKα, IKKβ and IKKγ in the gills of grass carp (Table 5). These results suggested that optimal levels of dietary protein might up-regulate IκBα to inhibit the nuclear translocation of NF-κB (NF-κB P65 and NF-κB P52) by down-regulating IKKα, IKKβ and IKKγ mRNA levels in the gills of fish.

Additionally, Weichhart et al. [71] reported that the mTOR signalling pathway is related to the modulation of anti-inflammatory cytokines gene expression in mammalian innate immune cells. As reported, S6K1 and 4E-BP are the downstream effectors of TOR in humans [72], and Guertin et al. [73] found that TOR activates S6K1 and inhibits 4E-BP expression in *Drosophila*. In human monocytes,

TOR increases production of the anti-inflammatory cytokines IL-10 [74]. In the present study, compared with low or high levels of protein, optimal levels of dietary protein led to the up-regulation of TOR and S6K1 mRNA levels and to the down-regulation of 4E-BP1 and 4E-BP2 mRNA levels in the gills of grass carp. Based on correlation analysis, the mRNA levels of anti-inflammatory cytokines IL-6, IL-11, TGF-β1 and TGF-β2 were positively correlated with the mRNA level of TOR in the gills of grass carp (Table 5), suggesting that optimal levels of dietary protein may partly through [TOR/(S6K1, 4E-BP1, 4E-BP2)] signalling to up-regulate these anti-inflammatory cytokines mRNA levels in the gills of fish. The fish physical barrier, composed of epithelial cells [17] and intercellular TJs [18], also plays a key role in maintaining the gill structural integrity. Therefore, we further explored the effects of dietary protein levels on the integrity of epithelial cells and their intercellular TJs in the gills of grass carp.

4.3. Optimal level of dietary protein improved physical barrier function in the gills of fish

4.3.1. Optimal level of dietary protein prevented oxidative damage and elevated antioxidant capacity partly through Nrf2 signalling pathway in the gills of fish

In fish, an excess of ROS causes oxidative damage to lipids and proteins, which is reflected in the contents of MDA and PC, respectively [75,76]. In the present study, compared with low or high levels of protein, at optimal levels of dietary protein, the contents of ROS, MDA and PC decreased in the gills of grass carp, indicating that an optimal level of dietary protein protected fish from oxidative damage to the gill. To our knowledge, oxidative damage is closely associated with non-enzymatic antioxidants such as GSH and antioxidant enzymes such as CAT and GPx in fish [77,78]. In the present study, compared with low or high levels of protein, at optimal dietary protein levels, the activities of CAT, GPx, GST and GR and the content of GSH were increased in the gills of grass carp, suggesting that at an optimal level of dietary protein the antioxidant capacity of fish gill improved.

We know that antioxidant enzyme activities are highly correlated with their mRNA levels in fish [79]. In this study, compared with low or high levels of protein, at optimal levels of dietary protein, the mRNA levels of CAT, GPx (GPx1a, 1b, 4a, 4b), GST (GSTR, O) and GR were up-regulated in the gills of grass carp. Correlation analysis revealed that the activities of CAT, GPx, GST and GR were positively correlated with their mRNA levels in the gills of grass carp (Table 5), suggesting that the increase in the activities of antioxidant enzymes at optimal levels of dietary protein might be attributed in part to the up-regulation of their mRNA levels in the gills of fish. Notably, at the optimal level of dietary protein, MnSOD activity decreased and CuZnSOD activity was not affected, although the mRNA levels of CuZnSOD and MnSOD were indeed up-regulated in the gills of grass carp. The different patterns observed between gene expression and their corresponding enzyme activities might be explained by two factors, in part. First, we observed no changes or decreases in enzyme activities because the increases in mRNA levels of antioxidant enzymes indicated an adaptive mechanism; the fish gill required more *de novo* synthesis of those antioxidant enzymes to scavenge excess ROS, but at either low or high levels of protein, the activities of those antioxidant enzymes were constantly inactivated by the ROS. Similar results were observed in previous studies in our laboratory with Jian carp (*Cyprinus carpio* var Jian) [80,81]. Second, the enzyme activities are influenced not only by gene transcription but also by post-transcriptional processes (such as translation and post-translational modification) in fish [80]. However, determination of the exact mechanism requires further investigation.

Moreover, Nrf2 is reported to be a key factor in promoting gene transcription of antioxidant enzymes in HepG2 cells [82]. In the present study, compared with low or high levels of protein, at the optimal level of dietary protein, the mRNA level of Nrf2 was up-regulated in the gills of grass carp. Based on correlation analysis, the mRNA levels of CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTR, GSTO and GR were positively correlated with the mRNA level of Nrf2 in the gills of grass carp (Table 5), suggesting that optimal level of dietary protein may partly through up-regulating Nrf2 translocation to up-regulate these antioxidant enzymes gene expression in the gills of fish. Meanwhile, Nrf2 constitutively binds to Keap1 in the cytoplasm of fish [83], and Velichkova and Hasson [84] demonstrated that knockdown of Keap1 gene increases Nrf2 activity in mice. In this study, compared with low or high levels of protein, at optimal levels dietary protein, the mRNA levels of Keap1a and Keap1b were down-regulated in the gills of grass carp. Based on correlation analysis, the mRNA level of Nrf2 was negatively correlated with the mRNA levels of Keap1a and Keap1b in the gills of grass carp (Table 5), suggesting that optimal level of dietary protein may promote Nrf2 translocation into the nucleus by down-regulating Keap1a and Keap1b mRNA levels in the gills of fish. Additionally, based on a previous study in our laboratory, oxidative damage induces apoptosis in the gills of grass carp [50]. Therefore, we further examined the effects of dietary protein levels on apoptosis in the gills of grass carp.

4.3.2. Optimal level of dietary protein inhibited apoptosis partly through JNK signalling molecules in the gills of fish

As generally acknowledged, caspases play a central role in apoptotic responses and are broadly divided into initiators, such as caspase 8 and caspase 9, and effectors, such as caspase 3 in Jurkat T lymphoblastoid cells [85]. In the present study, compared with low or high levels of protein, at optimal levels of dietary protein, the mRNA levels of caspase 3, caspase 8 and caspase 9 were down-regulated in the gills of grass carp, indicating that optimal level of dietary protein could inhibit apoptosis in fish gills. In humans, the two primary pathways to initiate apoptosis are the death receptor pathway and the mitochondrial pathway [86]. The death receptor pathway is primarily regulated by FasL and caspase 8 in rats [87], and the inhibition of FasL can decrease caspase 8 activity in Jurkat T lymphocytes [88]. In our study, compared with low or high levels of protein, at an optimal level of dietary protein, the mRNA level of FasL was down-regulated in the gills of grass carp. Based on correlation analysis, the mRNA level of caspase 8 was positively correlated with the mRNA level of FasL in the gills of grass carp (Table 5), suggesting that optimal level of dietary protein might be part through down-regulating FasL mRNA level to down-regulate caspase 8 mRNA level in the gills of fish. Additionally, inhibition of JNK suppresses FasL expression in human ovarian carcinoma cells [89]. In this study, compared with low or high levels of protein, at the optimal level of dietary protein, the mRNA level of JNK was down-regulated in the gills of grass carp. Based on correlation analysis, the mRNA level of FasL was positively correlated with that of JNK in the gills of grass carp (Table 5), suggesting that optimal level of dietary protein down-regulated FasL mRNA level might be partly related to the down-regulated mRNA level of JNK in the gills of fish. According to the above results, at different levels of dietary protein, JNK/FasL/caspase 8 signalling might regulate the death receptor apoptotic pathway in the gills of fish.

The Bcl-2 family includes anti-apoptotic members (such as Bcl-2 and Mcl-1) and pro-apoptotic members (such as Bax), which play key roles in the mitochondrial apoptotic pathway in humans [86]. Additionally, IAP inhibits caspases in mammals [90]. Inactivated Apaf-1 inhibits the activation of caspase 9, leading to the suppression of caspase 3 in human embryonic kidney 293T cells [91].

With the reported inhibition of pro-apoptotic Apaf-1 and Bax and the increase of anti-apoptotic Bcl-2, Mcl-1 and IAP suppress the cascade of reactions with the assistance of caspases in mammalian cells [92]. In the present study, compared with low or high levels of protein, at optimal levels of dietary protein, the mRNA levels of pro-apoptotic Apaf-1 and Bax were down-regulated and anti-apoptotic Bcl-2, Mcl-1 and IAP were up-regulated in the gills of grass carp. Based on correlation analysis, the mRNA level of caspase 3 was positively correlated with the mRNA levels of caspase 9, Apaf-1 and Bax, whereas the correlation was negative with Bcl-2, Mcl-1 and IAP mRNA levels in the gills of grass carp (Table 5), suggesting that the optimal level of dietary protein might be partly by down-regulating the mRNA levels of caspase 9, Apaf-1 and Bax and up-regulating the mRNA levels of Bcl-2, Mcl-1 and IAP to down-regulate the mRNA level of caspase 3 in the gills of fish. As these data indicate, at different levels of dietary protein, the mitochondrial apoptotic pathway might be partly regulated through (Apaf-1, Bax, Bcl-2, Mcl-1, IAP)/caspase 9/caspase 3 signalling in fish gills. We know TJs are an important part of the physical barrier for the fish gills [18]. Therefore, we next investigated the effects of dietary protein levels on the tight junctions in the gills of grass carp.

4.3.3. Optimal level of dietary protein strengthened tight junctions partly through MLCK and p38 MAPK signalling molecules in the gills of fish

In fish, the gill tight junction is composed primarily of TJs, such as occludin, ZO-1 and claudins [93]. The up-regulation of occludin, ZO-1 and claudin 12 mRNA levels revealed that damage to the gill epithelial TJ barrier function was attenuated in grass carp [94]. In the present study, compared with low or high levels of protein, at the optimal levels of dietary protein, we found that occludin, ZO-1, ZO-2, claudin c, claudin b, claudin 3, claudin 7a, claudin 7b, claudin 12, claudin 15a and claudin 15b mRNA levels were up-regulated in the gills of grass carp. These finding suggested that optimal level of dietary protein could enhance tight junction barrier in the gills of fish. In this case, at different levels of dietary protein, the regulation of the TJs in fish gills may be associated with MLCK. It was reported that inhibition of MLCK expression prevents the redistribution of occludin, ZO-1 and claudins, which improves tight junction barrier function in T84 cells [95]. In this study, compared with low or high levels of protein, at the optimal level of dietary protein, the gene expression of MLCK was down-regulated in the gills of grass carp. Based on correlation analysis, the mRNA levels of TJs (i.e. occludin, ZO-1, ZO-2, claudin c, claudin b, claudin 3, claudin 7a, claudin 7b, claudin 12, claudin 15a and claudin 15b) were negatively correlated with MLCK mRNA level in the gills of grass carp (Table 5). These results indicated that the up-regulation of TJ proteins in the gill at different levels of dietary protein was partly related to MLCK gene expression in fish. Moreover, MLCK gene expression is regulated by its upstream signalling molecule p38 MAPK, and the inhibition of p38 MAPK leads to the down-regulation of the MLCK gene expression in breast cancer cells [96]. In the present study, compared with low or high levels of protein, at the optimal level of dietary protein, the gene expression of p38 MAPK was down-regulated in the gills of grass carp. Based on correlation analysis, the mRNA level of MLCK was positively correlated with that of p38 MAPK in the gills of grass carp (Table 5), suggesting that optimal level of dietary protein down-regulated MLCK gene expression may be partly due to the down-regulation of p38 MAPK mRNA level in the gills of fish.

Of note in this study, the gene expression of ZO-2 and claudin 15b in the gills of grass carp were up-regulated at the optimal level of dietary protein. In previous studies in the intestine of this species of fish, we found that different levels of dietary protein had no significant effect on the mRNA levels of ZO-2 and claudin 15b [4]. Until this study, no information on the effect of dietary protein levels on ZO-2 gene expression in different tissues was available, and we hypothesized that the reasons were related to gene expression of IL-12p35. As reported, the inhibition of pro-inflammatory cytokine IL-12 production increases ZO-2 expression [97], and IL-12p35 is one of the subunits of IL-12 [98] in mice. In our studies, at the optimal level of dietary protein, IL-12p35 gene expression was down-regulated, but for the mRNA level of ZO-2, there was no significant change in the intestine. In contrast to the intestine, in the gill, at different levels of dietary protein, the mRNA level of IL-12p35 was not affected, whereas the gene expression of ZO-2 was up-regulated. Thus, the discrepancies in gene expression of ZO-2 in different tissues, as affected by dietary protein levels, might be due to IL-12p35. However, this possible mechanism requires further investigation. Additionally, the cause for the discrepancies in the data for claudin 15b from different tissues remains unclear, with two possible explanations for these discrepancies. First, the difference in the mRNA level of claudin 15b in grass carp gill versus intestine might be related to the transcript abundance in fish. Sun et al. [99] reported that the gene expression of claudin 15a is higher than that of claudin 15b in the intestine of fish, whereas the mRNA level of claudin 15a is similar to that of claudin 15b in the gills of channel catfish (*Ictalurus punctatus*). These data might explain the discrepancies in gene expression of claudin 15b between the intestine and gill in fish. Second, this discrepancy might be partly explained by the function of claudin 15. According to a literature report, claudin 15 creates Na^+-selective paracellular channels, which are associated with Na^+-K^+-ATPase in fish gills [100]. Meanwhile, a dietary protein supplement increases plasma growth hormone concentration in sea bream (*Sparus aurata*) [101], and McCormick [102] found that growth hormone enhanced the Na^+-K^+-ATPase activity in the gill of Atlantic salmon (*Salmo salar*). Hence, we hypothesize, at the optimal level of dietary protein, more Na^+ was required for transport than at low or high levels of protein and therefore, caused the up-regulation of claudin 15b in the gills, which did not occur in the intestine of fish. However, to test this hypothesis, further investigations are required.

4.4. Comparison of optimal levels of dietary protein for grass carp based on the different indices

In this study, we investigated optimal levels of dietary protein for grass carp based on different indices. The optimal level of dietary protein for the **against gill rot morbidity** in grass carp (264 g-787 g) was estimated to be 286.65 g kg^{-1} diet (253.73 g digestible protein kg^{-1} diet). Additionally, based on the **biochemical indices** [immune-related indices (ACP activity) and antioxidant-related indices (PC content)], the optimal levels of dietary protein for grass carp (264 g-787 g) were estimated to be 290.46 g kg^{-1} diet (257.76 g digestible protein kg^{-1} diet) and 296.25 g kg^{-1} diet (260.69 g digestible protein kg^{-1} diet), respectively. These optimal levels of dietary protein for grass carp (264 g-787 g) were based on disease resistance, and the immune-related and antioxidant-related indices were slightly higher than (or close to) that on the growth requirement 286.82 g kg^{-1} diet (250.66 g digestible protein kg^{-1} diet) evaluated in our previous study [4], suggesting that slightly more dietary protein was required to maintain gill health.

5. Conclusions

Overall, our study showed that an optimal level of dietary protein improved fish gill health, and we are also the first to reveal the possible mechanisms in fish gills. We found that at optimal levels of dietary protein, the production of antibacterial compounds increased and the gene expression of pro-inflammatory cytokines

down-regulated and that of anti-inflammatory cytokines up-regulated, in addition to an increase in antioxidant capacity, inhibited apoptosis and an improved tight junction barrier in the gills of grass carp after infection with *F. columnare*, leading to the increase of gill immune function and physical barrier function in fish. Moreover, the improvement in fish gill immune function and physical barrier function at optimal level of dietary protein might partially involve the signalling molecules NF-κB, TOR, p38 MAPK, MLCK, JNK and Nrf2. Additionally, based on the gill rot morbidity, ACP activity and PC content, the optimal levels of dietary protein for grass carp (264 g-787 g) were estimated to be 286.65 g kg^{-1} diet (253.73 g digestible protein kg^{-1} diet), 290.46 g kg^{-1} diet (257.76 g digestible protein kg^{-1} diet) and 296.25 g kg^{-1} diet (260.69 g digestible protein kg^{-1} diet), respectively.

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