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Threonine deficiency decreased intestinal immunity and aggravated inflammation associated with *NF-κB* and *target of rapamycin* signalling pathways in juvenile grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*

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Abstract

This study aimed to investigate the impacts of dietary threonine on intestinal immunity and inflammation in juvenile grass carp. Six iso-nitrogenous semi-purified diets containing graded levels of threonine (3·99–21·66 g threonine/kg) were formulated and fed to fishes for 8 weeks, and then challenged with *Aeromonas hydrophila* for 14 d. Results showed that, compared with optimum threonine supplementation, threonine deficiency (1) decreased the ability of fish against enteritis, intestinal lysozyme activities (except in the distal intestine), acid phosphatase activities, complement 3 (C3) and C4 contents and IgM contents (except in the proximal intestine (PI)), and it down-regulated the transcript abundances of *liver-expressed antimicrobial peptide* (*LEAP*)*-2A*, *LEAP-2B*, *hepcidin, IgZ, IgM* and *β-defensin1* (except in the PI) ($P < 0·05$); (2) could up-regulate intestinal pro-inflammatory cytokines *TNF-α*, *IL-1β*, *IL-6, IL-8* and *IL-17D* mRNA levels partly related to *NF-κB* signalling; (3) could down-regulate intestinal anti-inflammatory cytokine *transforming growth factor* (*TGF*)*-β*1, *TGF-β2*, *IL-4/13A* (not *IL-4/13B*) and *IL-10* mRNA levels partly by target of rapamycin signalling. Finally, on the basis of the specific growth rate, against the enteritis morbidity and IgM contents, the optimum threonine requirements were estimated to be 14·53 g threonine/kg diet (4·48 g threonine/100 g protein), 15.05 g threonine/kg diet (4·64 g threonine/100 g protein) and 15·17 g threonine/kg diet (4·68 g threonine/100 g protein), respectively.

Key words: Threonine deficiency: Intestine: Immunity: Inflammation: NF-κB and target of rapamycin signalling: Juvenile grass carp (*Ctenopharyngodon idella*)

Threonine is an essential amino acid for animals[(1)]. Previous studies from our laboratory observed that dietary threonine deficiency caused poor percentage weight gain (PWG), decreased feed efficiency (FE) and reduced digestive and brush border enzyme activities in fish intestines[(2,3)]. As we all know, the digestive and absorptive capacities of animals are correlated with intestinal health, which is closely related to intestinal immune function[(4)]. Intestinal immune function is dependent on its innate and adaptive immune responses[(5–8)]. However, there are just fragmentary reports about the effects of threonine on innate and adaptive immune responses in animal intestine. In animal intestine, it has been reported that threonine could promote the IgA production in broiler chicks[(9)], up-regulate inflammatory cytokine *IL-6* gene expression in piglets[(10)] and down-regulate genes expression of *IL-12* and *interferon γ* (*IFN-γ*) in broiler chicks[(11)], *IL-1β* in piglets[(10)] and *TNF-α* in blunt snout bream (*Megalobrama amblycephala*)[(12)]. However, those researches still lack systematicness, and they did not investigate

Abbreviations: 4E-BP1, eIF4E-binding protein 1; ACP, acid phosphatase; C, complement; DI, distal intestine; FE, feed efficiency; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-puruvate transaminase; IκBα, inhibitor of κBα; IKK, IκB kinase; INFγ, interferon γ; LA, lysozyme activities; LEAP, liver-expressed antimicrobial peptide; MI, mid intestine; PAC, plasma ammonia content; PI, proximal intestine; PWG, percentage weight gain; S6K1, ribosomal protein S6 kinases 1; SGR, special growth rate; TGF, transforming growth factor; TOR, target of rapamycin.

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the involved mechanisms. Therefore, systematic attempts to investigate the relationship between threonine and intestinal immune function and depth examination to explore the molecular mechanisms in animals are required.

Intestinal innate immune responses are related to its innate immune components such as antimicrobial peptides (such as *hepcidin*, *liver-expressed antimicrobial peptide* (*LEAP*)*-2A, LEAP-2B* and *β-defensin*), lysozyme activities (LA), acid phosphatase (ACP) and complements (such as C3 and C4), and the adaptive immune response depends on Ig production and T lymphocytes[13,14]. However, except for the reported Ig, no evidence was demonstrated about the effects of threonine on those innate immune components in animals. In the hepatopancreas of blunt snout bream, threonine could up-regulate the *peroxiredoxin II* (*Prx II*) mRNA levels[15]. It was confirmed that *Prx II* could increase the *hepcidin* expression in rat hepatocytes[16]. Meanwhile, threonine deficiency could depress the lymphocyte proliferation in the peripheral blood of piglets[10]. In addition, threonine deficiency could decrease the transcript abundance of *Complement C1 subcomponent precursor* (*C1S*) in the ileum of pigs[17]. Sarma & Ward[18] reported that *C1S* could prompt the generation of C3 in fish. As far as the above observations are concerned, we speculated that threonine might affect the intestinal immune function by modulating multiple innate immune components, which is worthy of systematic exploration.

Apart from the innate immune components, inflammatory cytokines also play a vital role in intestinal innate immune response, which consist of pro-inflammatory cytokines (such as *TNF-α* and *IL-1β*) and anti-inflammatory cytokines (such as *transforming growth factor* (*TGF*)*-β* and *IL-10*)[19,20]. Besides the studied cytokines (*TNF-α*, *IL-1β*, *IFN-γ*, *IL-6* and *IL-12*), other vital cytokines such as *IL-17D, IL-10, IL-4, IL-8* and *TGF-β* also participated in the important immune regulation in animals[21]. However, there is scarce information about the impacts of threonine on those various inflammatory cytokines (except *TNF-α*, *IL-1β*, *IFN-γ*, *IL-6* and *IL-12*) in animal intestine. In the lungs of weaned pigs, threonine could decrease relative mRNA abundance of *Toll-like receptor 9* (*TLR9*)[22]. Studies showed that *TLR9* could up-regulate the Treg *IL-17* and down-regulate the THP1 cell *IL-10* mRNA levels in humans [23,24]. Furthermore, pro-inflammatory cytokines could be regulated by *NF-κB* and *the inhibitor of κBα* (*IκBα*), as well as *IκB kinase* (*IKK*) complexes (*IKKα*, *IKKβ* and *IKKγ*)[25], whereas anti-inflammatory cytokines could be modulated by *mammalian target of rapamycin* (*mTOR*) in humans[26]. However, little evidence has been afforded about whether the effects of threonine on cytokines are related to the *NF-κB* (*p65/p52/c-Rel)/IκBα/IKK* and *mTOR* signalling regulation in animals. In blood monocytes of broilers, threonine deficiency up-regulated the nitric oxide level[27], which could induce the activation of *NF-κB* in rat heart[28]. In addition, Shibata *et al.*[29] reported that dietary threonine supplement decreased the nicotinamide level in rat brains. In mice, it was noteworthy that nicotinamide could lead to the suppression of *mTOR* in C17·2 neural stem cells[30]. The above observations indicate that there might be a potential relationship between threonine and multiple cytokines, as well as *NF-κB* and mTOR signalling, in animals, which needs to be investigated.

As reported, fish intestine displayed a regional immune specialisation in different intestines[31]. In the second gut segment of carp, more large resident intraepithelial Ig^+ macrophages can be found with a strong Ig-binding capacity[32]. Moreover, higher levels of T lymphocyte markers were observed in the posterior intestinal segment[33]. To our knowledge, threonine could contribute to the proliferation of lymphocytes in the weaned pigs[10]. Thus, it is of great value to investigate the effect of threonine on the intestinal immune response.

Grass carp (*Ctenopharyngodon idella*), a herbivorous finfish without a stomach, is the third biggest contributor to the worldwide aquaculture production[34,35]. As a kind of economic fish species, it accounts for about 16 % of the global freshwater aquaculture[36]. Recently, threonine requirement for juvenile grass carp was estimated on the basis of special growth rate (SGR)[37]. Nevertheless, nutrient requirements of fish may vary with different indices[38–42]. Therefore, it is of great value to estimate the threonine requirements of juvenile grass carp based on different indicators.

Together, on the basis of our previous studies that threonine deficiency decreased growth and reduced digestive and brush border enzyme activities in fish intestines, first of all, this study was systematically conducted to investigate the effects of threonine on the innate and adaptive immune components, as well as cytokines, in animal intestines after infection with *Aeromonas hydrophila*. In addition, further study was conducted, for the first time, to delve into the relationship between threonine and immune-related signalling molecules (*NF-κB* (*p65/p52/c-Rel)/IκBα/IKK* and *TOR/S6 kinase* (*S6K1*) and *eIF4E-binding protein 1* (*4E-BP1*)) with the aim of revealing the possible mechanism of threonine-regulating cytokines in animal intestines. Meanwhile, the threonine requirements of juvenile grass carp were also determined on the basis of different indicators, which may provide a reference for the commercial feed production of juvenile grass carp.

Methods

Experiments and designs

Composition of the basal diet is shown in Table 1. Fishmeal, casein and gelatin were used as dietary protein sources. Fish oil and soyabean oil were used as dietary lipid sources. The dietary protein level was fixed at 320·0 g/kg diet, as described by the National Research Council[43]. The overall composition of essential amino acids in the diets stimulated the amino acid pattern similar to that found in 320·0 g/kg crude protein from grass carp whole-body protein, excluding threonine, according to Wang *et al.*[44]. L-threonine was added to the basal diet to provide graded concentrations of 4·0 (un-supplemented diet), 7·5, 11·0, 14·5, 18·0 and 21·5 g threonine/kg diet. All diets were made iso-nitrogenous with graded glycine instead of incremental threonine according to Tang *et al.*[45]. Pellets were produced and stored at −20°C until use, as described by Hong *et al.*[3]. The threonine concentrations in the six experimental diets were analysed to be 3·99 (control), 7·70, 10·72, 14·10, 17·96 and 21·66 g threonine/kg diet according to Teshima *et al.*[46] using the Agilent 1100 series HPLC (Agilent Technologies).

Table 1. Composition and nutrient content of basal diet.

Ingredients	g/kg	Nutrient contents*	g/kg
Fishmeal	50·4	Crude protein	324·0
Casein	30·0	Crude lipid	32·6
Gelatin	78·6	*n*-3	5·0
Crystal amino acid mix†	203·1	*n*-6	10·0
Threonine premix‡	50·0	Available P	8·4
Fish oil	9·3		
Soyabean oil	19·4		
α-Starch	290·0		
Maize starch	150·5		
Ca $(H_2PO_4)_2$	33·2		
Vitamin premix§	20·0		
Mineral premix‖	10·0		
Choline chloride (60 %)	5·0		
Cellulose	50·0		
Ethoxyquin (30 %)	0·5		

* Crude protein and crude lipid contents were measured. Available P, *n*-3 and *n*-6 contents were calculated according to the National Research Council[43].

† Crystal amino acid mix (g/kg diet): lysine, 11·42; methionine, 7·81; tryptophan, 2·88; arginine, 9·56; histidine, 7·86; leucine, 18·64; isoleucine, 10·30; phenylalanine, 10·22; tyrosine, 6·44; valine, 11·25; cysteine, 2·87; glutamic acid, 57·50; glycine, 46·35, respectively.

‡ Threonine premix was added to obtain graded levels of threonine, and the amount of glycine and maize starch was reduced to compensate. Per kg of threonine premix composition from diets 1 to 6 was as follows (g/kg): L-threonine 0·00, 71·40, 142·80, 214·20, 285·80, 357·20; glycine 222·80, 178·40, 133·80, 89·20, 44·60, 0·00 and maize starch 777·20, 750·20, 723·40, 696·60, 669·60, 642·80, respectively.

§ Per kg of vitamin premix (g/kg): retinyl acetate (172 mg/g), 2·10; cholecalciferol (172 mg/g), 0·40; DL-*α*-tocopheryl 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 maize starch to 1 kg.

‖ Per kg of mineral premix (g/kg): $MnSO_4.H_2O$ (31·8 % Mn), 1·8900; $MgSO_4.H_2O$ (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_2SeO_3 (44·7 % Se), 0·0168. All ingredients were diluted with maize starch to 1 kg.

Experimental facility and fish husbandry

Fish husbandry was conducted in the University of Sichuan Agricultural Animal Care Advisory Committee. Juvenile grass carps were obtained from local fisheries (Sichuan, China). Fish were acclimated to the experimental environment for 4 weeks, as described by Hong *et al.*[3]. Then, 1080 fish with mean initial weights of 9·53 (SD 0·02) g were randomly assigned to eighteen experimental cages (1·4 L × 1·4 W × 1·4 H (m)), resulting in sixty juveniles per cage. Each cage was equipped with a disc of 100 cm diameter at the bottom to collect the uneaten feed, according to our laboratory study[47]. Each cage was randomly assigned to one of three replicates of the six dietary treatments, and fish were fed with the respective diet four times daily for 8 weeks, as described by Wen *et al.*[48]. A period of 30 min after feeding, uneaten feed was collected, dried and weighed to calculate the feed intake, as previously described by Hong *et al.*[3]. During the experiment, water temperature was 28 (SD 2) °C. The pH and dissolved O_2 levels were maintained at 7·0 (SD 0·2) mg/l and not <6·0 mg/l, respectively. Feeding trial was under natural light–dark cycle, similar to that described by Yue *et al.*[49].

Challenge trial and husbandry

According to our previous work, we used the successful model that was established by challenging with *A. hydrophila* and evaluating enteritis morbidity on the base of the severity of enteritis for estimating the enteritis resistance[50,51]. After the growth trial, sixty fish of similar body weight were obtained from each treatment group and moved to labelled cages for acclimating to the experimental condition for 5 d according to our laboratory study[50]. *A. hydrophila* was kindly provided by College of Veterinary Medicine, Sichuan Agricultural University, China. After the acclimatisation, fish were challenged with intraperitoneal injection of 1·0 ml of $2{\cdot}5 \times 10^5$ colony-forming units/ml *A. hydrophila* for each individual. The injection concentration was determined with a nonlethal dosage that could induce inflammation efficiently according to our preliminary test (data not shown). The challenge test lasted for 14 d according to Xu *et al.*[50] and our preliminary test. The experimental conditions during the *A. hydrophila* exposure trial were similar to those in the growth trial.

Sample collection

At the initiation and termination of the feeding trial, fish from each cage were weighed and counted, respectively. Thirty fish from the same population before the experiment and six fish from each treatment group at the end of the feeding trial were used for the determination of initial and final carcass proximate composition, as described by Feng *et al.*[2]. After the growth trial, 6 h after the last feeding, the blood samples of six fish from each treatment were drawn from the caudal vein, and then the plasma was removed and stored for analysis of plasma ammonia content (PAC), similar to the study by Chen *et al.*[52]. After that, forty-five fish from each treatment were randomly selected and anaesthetised in a benzocaine bath, as described by Geraylou *et al.*[53]. The fish were then killed, and the muscle and hepatopancreas of fish were quickly removed and frozen in N_2 and stored at −80°C, as described by Veiseth-Kent *et al.*[54], for later assay of the glutamate-oxaloacetate transaminase (GOT) and glutamate-puruvate transaminase (GPT) activities.

At the end of the challenge trial, fish from each treatment were anaesthetised as the same process as the growth trial. Then, the intestines of fish were quickly removed, segmented (proximal intestine (PI), mid intestine (MI) and distal intestine (DI)) and the severity of intestinal inflammation of fish was evaluated based on the method of Song *et al.*[55] and Refstie *et al.*[56]; the intestines were then frozen in N_2 and stored at −80°C for later analysis, as described by Deng *et al.*[47].

Biochemical parameter analysis

The approximate compositions of the feed and fish carcass were analysed according to the standard methods of the AOAC. PAC, GOT and GPT activities in hepatopancreas and muscle were assayed as described by Jiang *et al.*[57]. The intestinal samples were homogenised on ice in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at 6000 ***g*** at 4°C for 20 min, and then the collected supernatant was stored for the subsequent analysis of related parameters, as described by Chen *et al.*[52]. The intestine LA and ACP activity were determined according to the method of El-Boshy *et al.*[58] and Molina *et al.*[59], respectively. The contents of C3 and C4 were measured by using the immunoturbidimetry kit (Nanjing Jiancheng Bioengineering Institute), according to the method of Zhang

et al.[60]. The IgM content was analysed by using the immunoturbidimetry kit (Nanjing Jiancheng Bioengineering Institute), according to the method of Li *et al.*[61].

Real-time PCR analysis

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were similar to those descriptions conducted in the previous study in our laboratory[48]. The total RNA of the samples was extracted from the PI, MI and DI using the RNAiso Plus kit (TaKaRa) according to the manufacturer's instructions, followed by DNAse I treatment. The total 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 complementary DNA (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 in the National Center for Biotechnology Information (NCBI) (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 normalise cDNA loading. The target and housekeeping gene amplification efficiencies were calculated according to the specific gene standard curves generated from 10-fold serial dilutions. The $2^{-\Delta\Delta C_t}$ method was used to calculate the expression results after verifying that the primers amplified with an efficiency of approximately 100%, as described by Livak & Schmittgen[62].

Western blot analysis

The protein homogenate preparation from intestines, antibodies and western blotting were processed as described in our previous studies[63,64]. We determined the protein concentrations using a BCA assay kit (Beyotime Biotechnology Inc.). Protein samples (40 μg/lane) were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane for western blot analysis. The membrane was blocked for 1 h at room temperature and then incubated with primary antibody overnight at 4°C. We used the same anti-total TOR, p-TOR Ser 2448 and β-Actin antibodies as those described in our previous studies[63,64]. β-Actin was used as control proteins for total protein. After being washed, the PVDF membrane was incubated for 2 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in TBST (Tris-buffered saline, with Tween-20). The immune complexes were visualised using ECL reagents (Beyotime Biotechnology Inc.). The western blot bands were quantified using the NIH Image 1.63 software. Different treatments were expressed relative to the level of the control group. This experiment was repeated at least three times, and similar results were obtained each time.

Calculations and statistical analysis

Growth performance parameters were calculated on the basis of the following formulas: growth performance was assessed in

Table 2. Real-time PCR primer sequences

Target genes	Primer sequence (forward (5′ → 3′))	Primer sequence (reverse (5′ → 3′))	Temperature (°C)	Accession no.
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59·3	JQ246442·1
LEAP-2A	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59·3	FJ390414
LEAP-2B	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59·3	KT625603
β-Defensin1	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58·4	KT445868
IgM	CGATGCTTTTGACTACTGGGGA	AGAAGAACACTGAGACAGGGCG	57·1	DQ417927
IgZ	CCAGTCAGTCCAGGGAAGG	GTAGTCAAAGGCAGCCGTCAG	58·4	GQ201421
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-12p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55·4	KF944667·1
IL-12p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	59·0	KF944668·1
IL-17D	GTGTCCAGGAGAGCACCAAG	GCGAGAGGCTGAGGAAGTTT	62·3	KF245426·1
IL-4/13A	CTACTGCTCGCTTTCGCTGT	CCCAGTTTTCAGTTCTCTCAGG	55·9	KT445871
IL-4/13B	TGTGAACCAGACCCTACATAACC	TTCAGGACCTTTGCTGCTTG	55·9	KT625600
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
c-Rel	GCGTCTATGCTTCCAGATTTACC	ACTGCCACTGTTCTTGTTCACC	59·3	KT445865
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
β-Actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	61·4	M25013

LEAP-2, liver-expressed antimicrobial peptide 2; *IFN-γ2*, interferon γ2; *TGF-β*, transforming growth factor β; *IκBα*, inhibitor of κBα; *IKK*, IκB kinase; *TOR*, target of rapamycin; *S6K1*, ribosomal protein S6 kinases 1; *4E-BP1*, eIF4E-binding protein 1.

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terms of PWG, SGR, feed intake (FI) and FE, protein efficiency ratio (PER) and protein retention value (PRV):

$$\text{PWG}(\%) = 100 \times (\text{final weight}(\text{g/fish}) - \text{initial weight}(\text{g/fish}) / \text{initial weight}(\text{g/fish})$$

$$\text{SGR}(\%) = 100 \times (\ln(\text{final weight}) - \ln(\text{initial weight})) / \text{d},$$

$$\text{FE}(\%) = 100 \times (\text{final weight}(\text{g/fish}) - \text{initial weight}(\text{g/fish})) / \text{FI},$$

$$\text{PER} = \text{g weight gain} / \text{g protein intake},$$

$$\text{PRV} = (\text{final total body protein} - \text{initial body protein}) / \text{total protein intake}.$$

All data were subjected to a one-way ANOVA followed by the Duncan's multiple-range test to evaluate significant differences among treatments at $P < 0{\cdot}05$ with SPSS 18.0 (SPSS Inc.), as described by Jiang *et al.*[65]. On the basis of the means and standard deviations of growth and intestinal immune-related parameters, the minimum effect size was calculated to be 0·67 according to the method of Searcy-Bernal[66]. With the effect size of 0·67, a significance level of 0·05 and the six replicates in each treatment, the statistical power was calculated to be 0·82 using the R pwr package according to Grey *et al.*[67]. On the basis of the SGR and intestinal health indicators, the threonine requirements were estimated by quadratic regression model according to the method of Ahmed *et al.*[68].

Results

Growth performance of juvenile grass carp, glutamate-oxaloacetate transaminase and glutamate-puruvate transaminase activities in the muscle and hepatopancreas, plasma ammonia content and enteritis morbidity

Effects of graded threonine levels on juvenile grass carp growth parameters are presented in Table 3. The growth performance (IBW, FBW, PWG, SGR, FI, FE, PER and PPV) was elevated significantly ($P < 0{\cdot}05$) with increased threonine levels up to 14·10 g threonine/kg diet, and then decreased significantly ($P < 0{\cdot}05$). As shown in Table 4, the GOT and GPT activities of the hepatopancreas were enhanced significantly with added threonine levels up to 14·10 g threonine/kg diet ($P < 0{\cdot}05$), and then decreased gradually. Meanwhile, with the addition of dietary threonine up to 14·10 g threonine/kg diet, increased muscle activities were obtained significantly in the GOT and GPT ($P < 0{\cdot}05$), and then decreased significantly ($P < 0{\cdot}05$) in the GOT but slightly in the GPT, respectively. Besides, the PAC decreased significantly with threonine level up to 14·10 g threonine/kg diet ($P < 0{\cdot}05$), and then increased significantly ($P < 0{\cdot}05$). In addition, with threonine level up to 14·10 g threonine/kg diet, enteritis morbidity after challenge with *A. hydrophila* was abated significantly ($P < 0{\cdot}05$) and then increased gradually (Fig. 1 and 2).

Intestine immune parameters

The intestinal activities of the LA, ACP and the contents of C3 and C4 in the three intestinal segments of juvenile grass carp after infection with *A. hydrophila* are presented in Table 5. With the addition of threonine up to 14·10 and 17·96 g threonine/kg diet individually, the LA enhanced significantly ($P < 0{\cdot}05$) in the PI but slightly in the MI, and then all decreased gradually. With the addition of threonine up to 14·10 g threonine/kg diet, the contents of C3 in the DI, IgM in the MI and DI increased gradually, and then all decreased smoothly. With the addition of threonine up to 14·10, 10·72 and 10·72 g threonine/kg diet, the ACP activities in the PI and the C3 contents in the PI and MI increased significantly ($P < 0{\cdot}05$), and then all plateaued ($P > 0{\cdot}05$). Compared with the dietary threonine deficiency, threonine supplementation increased the activities of the ACP in the MI and DI, and the C4 contents in the PI and MI. Meanwhile, fish fed 10·72 g threonine/kg diet showed the maximum C4 contents in the DI. On the contrary, the LA in the DI were diminished with threonine level up to 10·72 g threonine/kg diet ($P < 0{\cdot}05$), and then plateaued ($P > 0{\cdot}05$). However, dietary threonine had no effects on the IgM contents in the PI ($P > 0{\cdot}05$).

Gene expression in the intestine

Relative expressions of innate and adaptive components and inflammatory cytokines mRNA in fish intestines. The effects of dietary threonine on innate and adaptive components

Table 3. Initial body weight (IBW), final body weight (FBW), percentage weight gain (PWG), feed intake (FI), feed efficiency (FE), specific growth rate (SGR), protein efficiency ratio (PER) and protein retention value (PRV) of juvenile grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of threonine for 8 weeks*
(Mean values and standard deviations)

	3·99		7·70		10·72		14·10		17·96		21·66	
Thr levels (g/kg diet)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IBW (g)	9·51^{a}	0·03	9·53^{a}	0·01	9·53^{a}	0·03	9·55^{a}	0·01	9·54^{a}	0·03	9·54^{a}	0·04
FBW (g)	26·19^{a}	0·57	36·33^{b}	0·88	44·91^{c}	1·09	53·43^{d}	1·48	45·52^{c}	1·51	37·14^{b}	1·63
PWG (%)	175·32^{a}	5·54	281·42^{b}	8·86	372·03^{c}	11·86	459·53^{d}	17·03	377·00^{c}	15·40	289·07^{b}	15·68
SGR (%/d)	1·81^{a}	0·04	2·39^{b}	0·04	2·77^{c}	0·04	3·07^{d}	0·05	2·79^{c}	0·06	2·43^{b}	0·07
FI (g)	29·37^{a}	0·10	44·06^{b}	0·76	52·18^{c}	0·30	59·81^{d}	0·34	52·91^{c}	1·04	44·36^{b}	0·28
FE (%)	56·78^{a}	1·97	60·83^{a,b}	0·95	67·83^{c}	1·73	73·77^{d}	2·61	68·38^{c}	2·64	62·19^{b}	3·36
PER	1·75^{a}	0·06	1·88^{b}	0·03	2·09^{c}	0·05	2·25^{d}	0·07	2·11^{c}	0·06	1·92^{b}	0·10
PRV	24·33^{a}	1·20	26·24^{b}	1·06	30·43^{d}	1·38	33·94^{e}	1·90	28·69^{c,d}	1·62	27·03^{b,c}	2·20

^{a,b,c,d,e} Mean values in the same row with unlike superscripts were significantly different ($P < 0{\cdot}05$; ANOVA and Duncan's multiple-range tests).
* Values for IBW, FBW, PWG, SGR, FI and FE of three replicates groups, with sixty fish in each group. Values for PER and PRV of six replicates.

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Table 4. Glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activities in muscle and hepatopancreas; plasma ammonia contents (PAC) of juvenile grass carp (*Ctenopharyngodon idella*) fed with diets containing graded levels of threonine for 8 weeks (Mean values and standard deviations, six replicates)

	3·99		7·70		10·72		14·10		17·96		21·66	
Thr levels (g/kg diet)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
GOT activities (U/g tissue)												
Muscle	2063·7^{a}	92·4	2993·2^{b}	224·2	3676·9^{c}	129·9	4215·5^{d}	259·3	3645·3^{c}	105·3	3058·4^{b}	276·7
Hepatopancreas	4199·6^{a}	217·3	5533·6^{b}	355·0	6296·5^{c}	212·3	6694·1^{d}	335·2	6159·5^{c}	259·0	5968·3^{c}	179·2
GPT activities (U/g tissue)												
Muscle	899·5^{a}	57·0	1168·0^{b}	89·1	1323·7^{c,d}	111·5	1489·8^{e}	119·7	1402·0^{d,e}	58·4	1295·5^{c}	40·1
Hepatopancreas	1136·5^{a}	77·3	1471·1^{b}	93·7	1731·3^{c}	83·2	1968·3^{d}	76·3	1893·0^{d}	143·6	1745·2^{c}	145·5
Ammonia contents (μmol/l)												
Plasma	400·4^{e}	16·2	290·9^{d}	10·8	258·7^{c}	18·1	200·2^{a}	4·2	225·4^{b}	12·5	284·6^{d}	8·1

^{a,b,c,d,e} Means values in the same row with unlike superscripts are significantly different ($P<0·05$; ANOVA and Duncan's multiple-range tests).

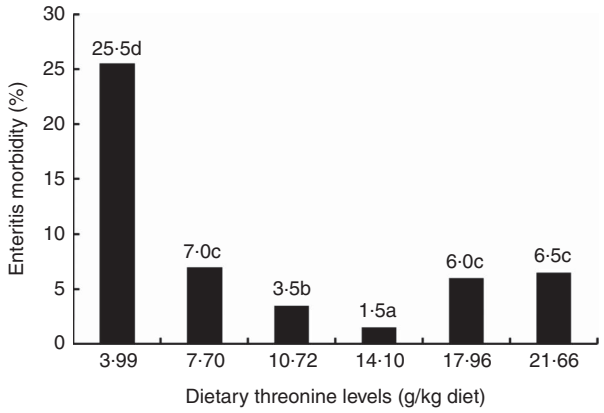

Fig. 1. The enteritis morbidity of juvenile grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of threonine after infection with *Aeromonas hydrophila* for 14 d. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P<0·05$; ANOVA and Duncan's multiple-range tests).

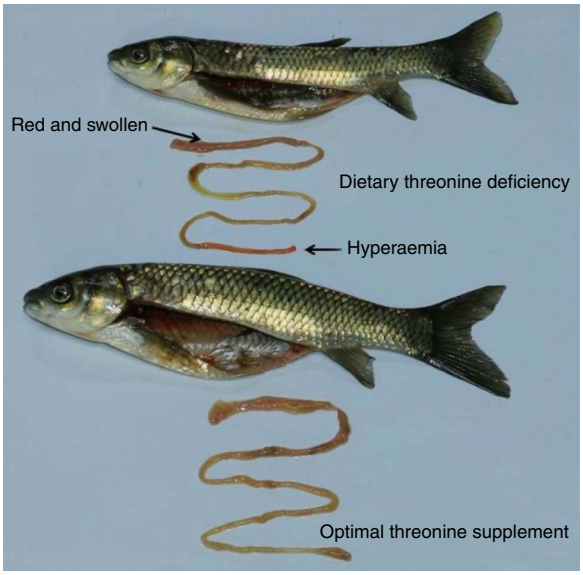

Fig. 2. Compared with optimal threonine supplementation, threonine deficiency led to obviously enteritis symptom of juvenile grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of threonine for 8 weeks after infection with *Aeromonas hydrophila* for 14 d. Visibly red and swollen and hyperaemia were observed for fish fed the threonine-deficient diet after infection with *A. hydrophila*.

and inflammatory cytokines in the three intestinal segments of fish after infection with *A. hydrophila* are presented in Fig. 3 and 4. In the PI, with the addition of threonine up to 14·10, 14·10 and 14·10 g threonine/kg diet, the mRNA levels of *hepcidin*, *LEAP-2A* and *IL-10* were up-regulated, respectively, and then all down-regulated gradually. Meanwhile, compared with threonine deficiency, optimal threonine supplementation up-regulated the *LEAP-2B, IgZ, TGF-β1, TGF-β2* and *IL-4/13A* mRNA levels significantly ($P<0·05$). However, there were no marked differences between the threonine-deficient diet group (3·99 g threonine/kg diet) and any other group added graded levels of threonine on *β-defensin1* and *IgM* mRNA levels ($P>0·05$). In the MI, with the addition of threonine up to 10·72, 14·10, 14·10, 14·10, 14·10 and 14·10 g threonine/kg diet, the mRNA levels of *hepcidin, LEAP-2A*, *β-defensin1, IgZ, TGF-β1* and *TGF-β2* were elevated individually, and then all decreased gradually. The mRNA levels of *LEAP-2B*, *IgM*, *IL-4/13A* and *IL-10* in the MI were lowest for juveniles fed a threonine-deficient diet ($P<0·05$), individually. In the DI, the mRNA levels of *hepcidin, LEAP-2A, LEAP-2B*, *β-defensin1, IgM, IgZ, TGF-β2*, *IL-4/13A* and *IL-10* were up-regulated individually with increased threonine levels up to 14·10 g threonine/kg diet, and then all down-regulated gradually. Fish fed a threonine-deficient diet showed the minimum *TGF-β1* level in the DI of juvenile grass carp ($P<0·05$). Surprisingly, no remarkable differences were found in the *IL-4/13B* mRNA levels in the three intestinal segments between fish fed graded levels of threonine ($P>0·05$).

In addition, in the PI, the mRNA levels of *TNF-α*, *IFN-γ2* and *IL-17D* were down-regulated individually with the addition of threonine up to 14·10, 14·10 and 10·72 g threonine/kg diet, and then all up-regulated, gradually. Compared with dietary threonine supplementation, threonine deficiency up-regulated the *IL-1β*, *IL-6* and *IL-8* mRNA levels, significantly ($P<0·05$). In the MI, the mRNA levels of *TNF-α*, *IL-1β*, *IFN-γ2* and *IL-17D* were down-regulated with the addition of threonine up to 14·10, 10·72, 10·72 and 10·72 g threonine/kg diet, respectively, and then all up-regulated gradually. Meanwhile, fish fed the threonine deficiency diet showed the highest *IL-6* and *IL-8* mRNA levels ($P<0·05$). In the DI, the mRNA levels of *TNF-α*, *IL-8* and *IL-17D* were down-regulated individually with the addition of dietary threonine up to 10·72, 14·10 and 14·10 g threonine/kg diet, and then all up-regulated, gradually. Compared with threonine supplementation, threonine deficiency enhanced the *IL-1β*, *IFN-γ2*

Table 5. Immune components in the intestine of juvenile grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of threonine for 8 weeks after injection with *Aeromonas hydrophila* for 14 d
(Mean values and standard deviations)

	3·99		7·70		10·72		14·10		17·96		21·66	
Thr levels (g/kg)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lysozyme activities (U/mg protein)												
PI	149·40^{a}	9·32	187·75^{b}	17·92	211·78^{c}	19·82	232·44^{d}	18·27	207·09^{b,c}	20·11	200·09^{b,c}	11·24
MI	57·35^{a}	3·62	114·31^{b,c}	9·06	107·32^{b}	4·62	113·80^{b,c}	6·07	117·03^{c}	7·36	112·91^{b,c}	6·34
DI	215·74^{c}	18·31	172·18^{b}	12·20	112·90^{a}	8·76	110·47^{a}	6·35	108·34^{a}	5·87	109·59^{a}	7·68
Acid phosphatase activities (U/mg protein)												
PI	123·92^{a}	11·55	180·36^{b}	13·64	229·62^{c}	18·73	284·94^{d}	18·47	281·16^{d}	27·25	273·06^{d}	10·14
MI	111·45^{a}	4·55	130·78^{b}	12·58	138·32^{b}	7·17	137·65^{b}	5·77	134·51^{b}	7·12	138·47^{b}	8·11
DI	87·50^{a}	5·16	114·79^{b}	5·58	123·55^{b}	8·90	117·47^{b}	7·78	117·27^{b}	5·27	119·35^{b}	7·14
Complement 3 contents (mg/g protein)												
PI	11·88^{a}	0·86	13·75^{b}	0·87	16·12^{c}	0·90	16·06^{c}	0·60	15·11^{c}	0·93	15·22^{c}	0·77
MI	8·22^{a}	0·65	10·77^{b}	0·85	11·75^{c}	0·67	12·19^{c}	0·50	12·05^{c}	0·81	12·06^{c}	0·73
DI	11·14^{a}	0·90	13·45^{b}	0·64	13·53^{b}	1·08	14·87^{c}	0·51	13·39^{b}	1·10	13·37^{b}	0·83
Complement 4 contents (mg/g protein)												
PI	1·14^{a}	0·11	1·62^{b}	0·11	1·61^{b}	0·14	1·70^{b}	0·14	1·63^{b}	0·16	1·68^{b}	0·08
MI	1·35^{a}	0·04	1·62^{b}	0·16	1·66^{b}	0·15	1·68^{b}	0·16	1·77^{b}	0·17	1·62^{b}	0·15
DI	1·28^{a}	0·11	1·36^{a}	0·16	1·91^{b}	0·15	1·90^{b}	0·14	1·79^{b}	0·14	1·85^{b}	0·18
IgM contents (mg/g protein)												
PI	42·48^{a}	3·68	41·70^{a}	2·83	42·77^{a}	3·13	41·59^{a}	3·85	44·39^{a}	3·07	41·82^{a}	3·34
MI	56·85^{a}	4·29	66·63^{b}	4·47	69·38^{b}	1·48	75·26^{c}	3·44	69·65^{b}	2·54	68·63^{b}	4·88
DI	66·61^{a}	4·48	74·67^{b}	4·75	82·25^{b,c}	4·37	83·51^{c}	7·49	76·70^{b,c}	6·11	75·65^{b}	7·48

PI, proximal intestine; MI, mid intestine; DI, distal intestine.
^{a,b,c,d} Mean values in the same row with unlike superscripts were significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

and *IL-6* mRNA levels significantly ($P < 0·05$). Characteristically, dietary threonine had no effects on the mRNA levels of *IL-12 p35* and *IL-12 p40* in the three intestinal segments of juvenile grass carp ($P > 0·05$).

Relative expressions of immune-related signal molecules in the intestines of fish. The effects of threonine on relative expressions of the *NF-κB p65, NF-κB p52, c-Rel, IκBα, IKKα, β, γ, TOR, p70 S6K1* and *4E-BP1* after infection with *A. hydrophila* are presented in Fig. 5. In the PI, the addition of threonine up to 14·10, 10·72, 14·10, 14·10 and 14·10 g threonine/kg diet down-regulated mRNA levels significantly in the *IKKβ* ($P < 0·05$) and slightly in the *NF-κB p65*, *NF-κB p52*, *IKKγ* and *4E-BP1*, and then all up-regulated gradually. On the contrary, the addition of threonine up to 14·10 g threonine/kg diet up-regulated the mRNA levels in the *TOR, IκBα* and *S6K1*, and then all down-regulated gradually. In the MI, the mRNA levels of *NF-κB p52*, *IKKβ*, *IKKγ* and *4E-BP1* were down-regulated with threonine levels up to 14·10, 14·10, 14·10 and 17·96 g threonine/kg diet, respectively, and then all up-regulated gradually. Besides, the *TOR* mRNA levels were up-regulated with threonine levels added up to 14·10 g threonine/kg diet, and then down-regulated gradually. Besides, fish fed the threonine deficiency diet showed the maximum mRNA level in *NF-κB p65* and the minimum in *IκBα* and *S6K1* ($P < 0·05$). In the DI, the addition of threonine up to 14·10 g threonine/kg diet down-regulated the mRNA levels slightly in the *NF-κB p52*, *NF-κB p65*, *IKKβ*, *IKKγ* and *4E-BP1*, and then all up-regulated progressively. Meanwhile, *IκBα* and *TOR* mRNA levels were up-regulated individually with dietary threonine levels up to 14·10 and 17·96 g threonine/kg diet, and then all down-regulated gradually. Compared with threonine supplementation, threonine deficiency down-regulated *S6K1* mRNA level significantly ($P < 0·05$). Interestingly, no marked influences were detected in the *IKKα* and *c-Rel* mRNA levels in the three intestinal segments of juvenile grass carp ($P > 0·05$).

Protein levels and phosphorylation of target of rapamycin in the intestine of fish

The effects of threonine on the protein levels of total TOR (T-TOR) and phosphorylation of TOR on residue Ser2448 (p-TOR Ser2448) after infection with *A. hydrophila* are presented in Fig. 6. With the addition of threonine up to 17·96 g threonine/kg diet, the protein levels of the T-TOR increased gradually in the PI, MI and DI, and then decreased gradually in the DI and significantly in the PI and MI of juvenile grass carp ($P < 0·05$). With the addition of threonine up to 17·96, 10·72 and 14·10 g threonine/kg, respectively, enhanced protein levels of the p-TOR Ser2448 were found in the PI, MI and DI, and then all decreased significantly in the PI ($P < 0·05$) and gradually in the MI and DI of juvenile grass carp.

Discussion

Threonine deficiency decreased fish growth performance and the enteritis resistance

According to the study, dietary threonine deficiency decreased the growth performance of juvenile grass carp with poor PWG, FI, FE, SGR, PER and PRV. On the basis of the quadratic regression analysis of the SGR, the optimal dietary threonine level for juvenile grass carp was estimated to be 14·53 g threonine/kg diet (4·48 g threonine/100 g protein). Meanwhile, accelerated growth performance was associated with improvement of amino acid utilisation, which reflected in depressed PAC[69] and enhanced GOT and GPT levels in the hepatopancreas and muscle[70]. Our study displayed that dietary threonine deficiency increased the PAC and decreased

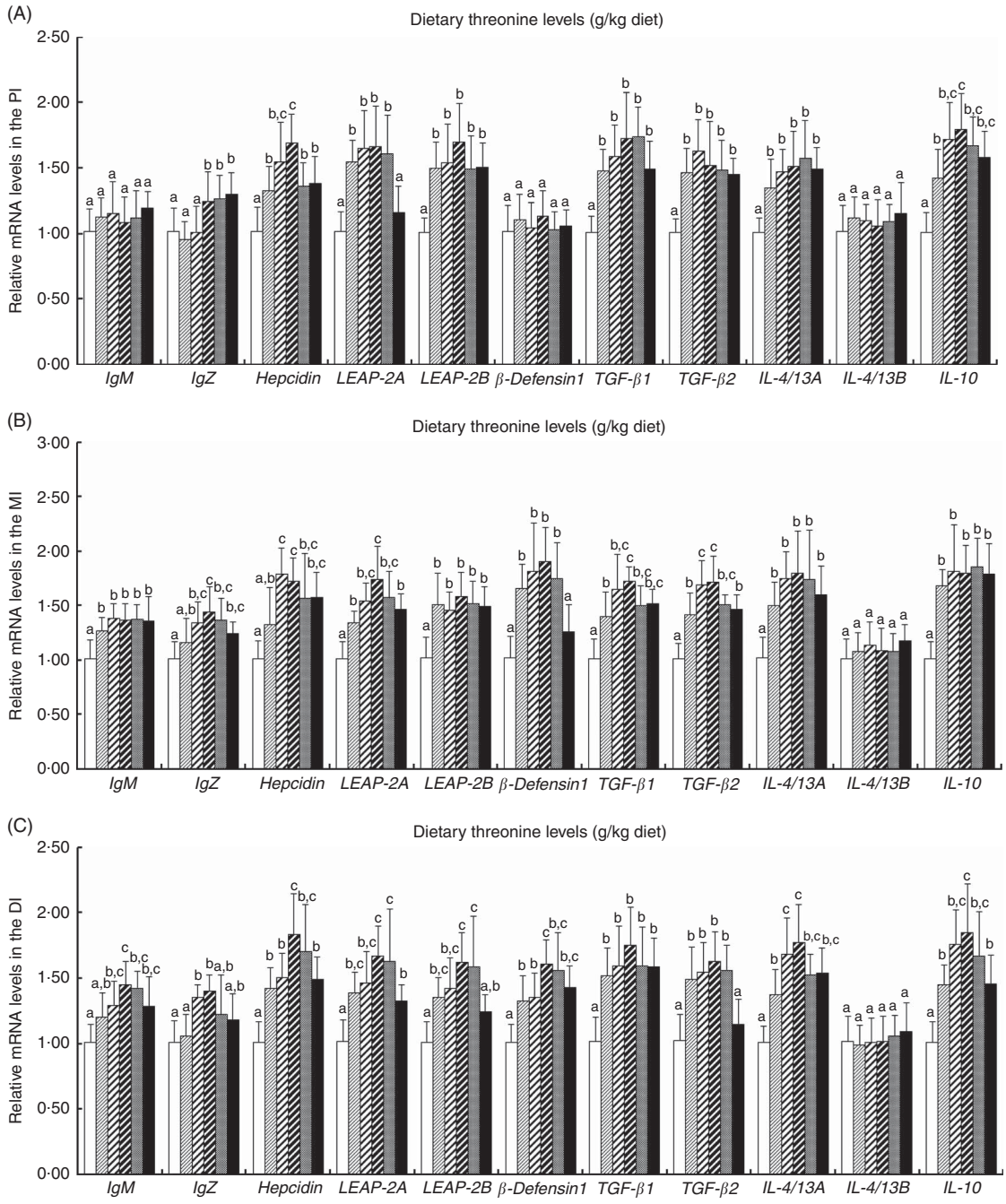

Fig. 3. Relative expression of *IgM, IgZ, hepcidin, liver-expressed antimicrobial peptide* (*LEAP*)*-2A, LEAP-2B* and *β-defensin1* and anti-inflammatory cytokines (*TGF-β1*, *TGF-β2*, *IL-4/13A, IL-4/13B* and *IL-10*) in the proximal intestine (PI, A), middle intestine (MI, B) and distal intestine (DI, C) of fish fed diets containing graded levels of threonine for 8 weeks after infection with *Aeromonas hydrophila* for 14 d. Values are means (six fishes per group), and standard deviations represented by vertical bars. □, Thr 3·99; ▨, Thr 7·70; ▨, Thr 10·72; ▨, Thr 14·10; ▨, Thr 17·96; ■, Thr 21·66. [a,b,c] Mean values with unlike letters were significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

the GOT and GPT activities in muscle and hepatopancreas of juvenile grass carp. In addition, it is well known that fish growth is related to intestine health, which is partly reflected in the enteritis resistance[71]. Previous studies of our laboratory demonstrated that the higher enteritis morbidity could reflect the weaker enteritis resistance after fish were challenged with *A. hydrophila*[50,60]. Thus, we next investigated the effect of threonine on the resistance to enteritis of fish after challenging them with *A. hydrophila*.

In the present study, we demonstrated for the first time that, compared with dietary threonine deficiency resulting in the maximum enteritis morbidity (25·5 %), the optimal threonine level significantly decreased the enteritis morbidity to be 1·5 % in juvenile grass carp after infection with *A. hydrophila* ($P < 0·05$), indicating that dietary threonine deficiency attenuated the resistance ability against enteritis in fish. On the basis of the enteritis morbidity, the optimal threonine level to reinforce resistance against enteritis was recommended to be 15·05 g

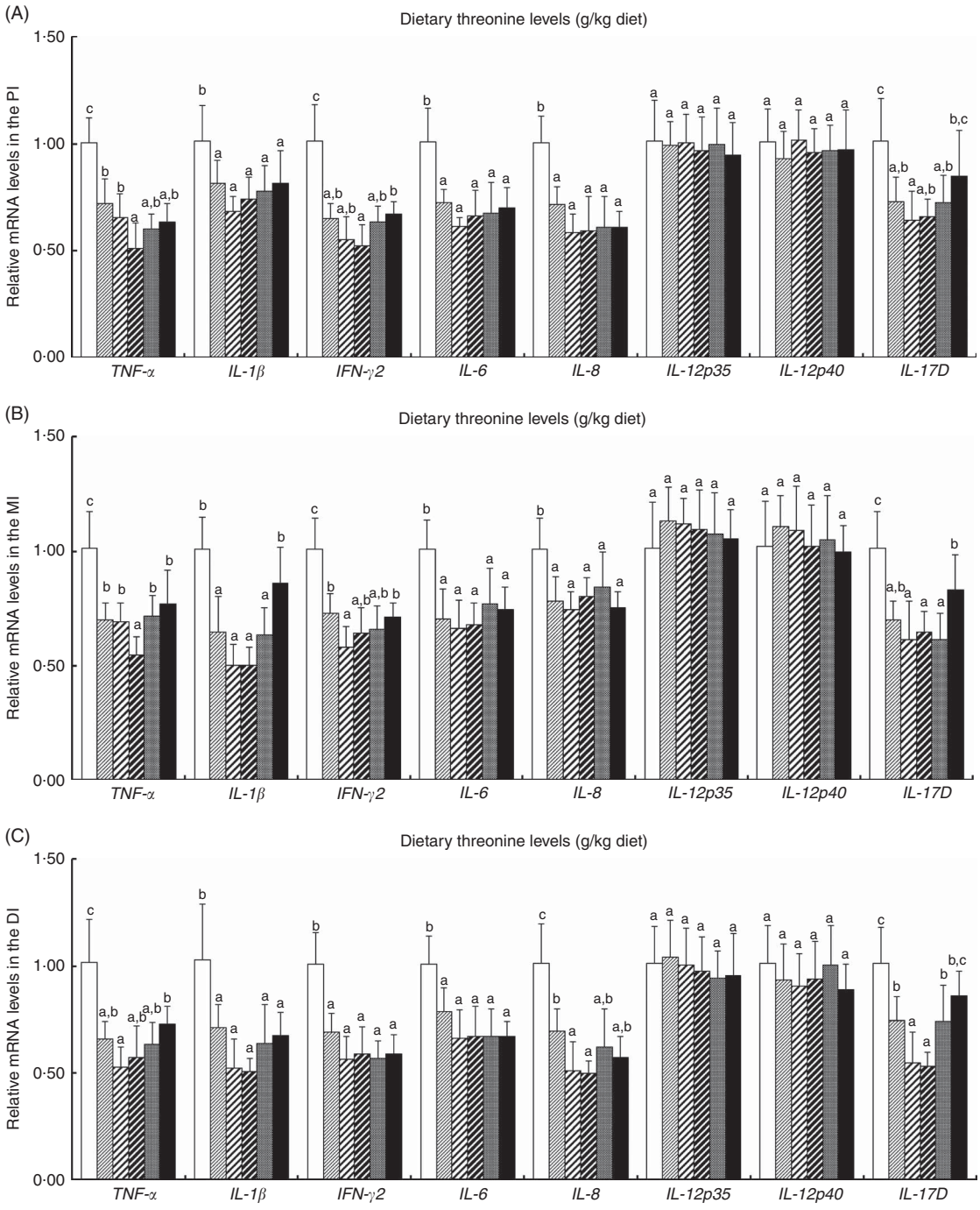

Fig. 4. Relative expression of pro-inflammatory cytokines (*TNF-α*, *IL-1β*, *IFN-γ2*, *IL-6*, *IL-8*, *IL-12p35*, *IL-12p40*, and *IL-17D*) in the proximal intestine (PI) (A), middle intestine (MI) (B) and distal intestine (DI) (C) of fish fed diets containing graded levels of threonine for 8 weeks after infection with *Aeromonas hydrophila* for 14 d. Values are means (six fishes per group), and standard deviations represented by vertical bars. □, Thr 3·99; ▨, Thr 7·70; ▨, Thr 10·72; ▨, Thr 14·10; ▩, Thr 17·96; ■, Thr 21·66. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

threonine/kg diet (4·64 g threonine/100 g protein) ($Y = 0·1841x^2 - 5·5399x + 42·4060$, $R^2 = 0·897$, $P < 0·05$), which was slightly higher than that based on SGR (14·53 g threonine/kg diet). In addition, enteritis resistance is related to intestinal immune function, which is partly dependent on the innate and adaptive responses in fish[50,71]. Thus, we next investigated the impacts of dietary threonine on innate and adaptive responses in the intestines of juvenile grass carp after infection with *A. hydrophila*.

Threonine deficiency impaired fish intestinal immunity by decreasing innate and adaptive immune component production after infection with Aeromonas hydrophila

In this study, for the first time, we investigated the effects of threonine on innate and adaptive immune components in fish intestines after infection with *A. hydrophila*. Current results displayed that dietary threonine deficiency decreased LA in PI

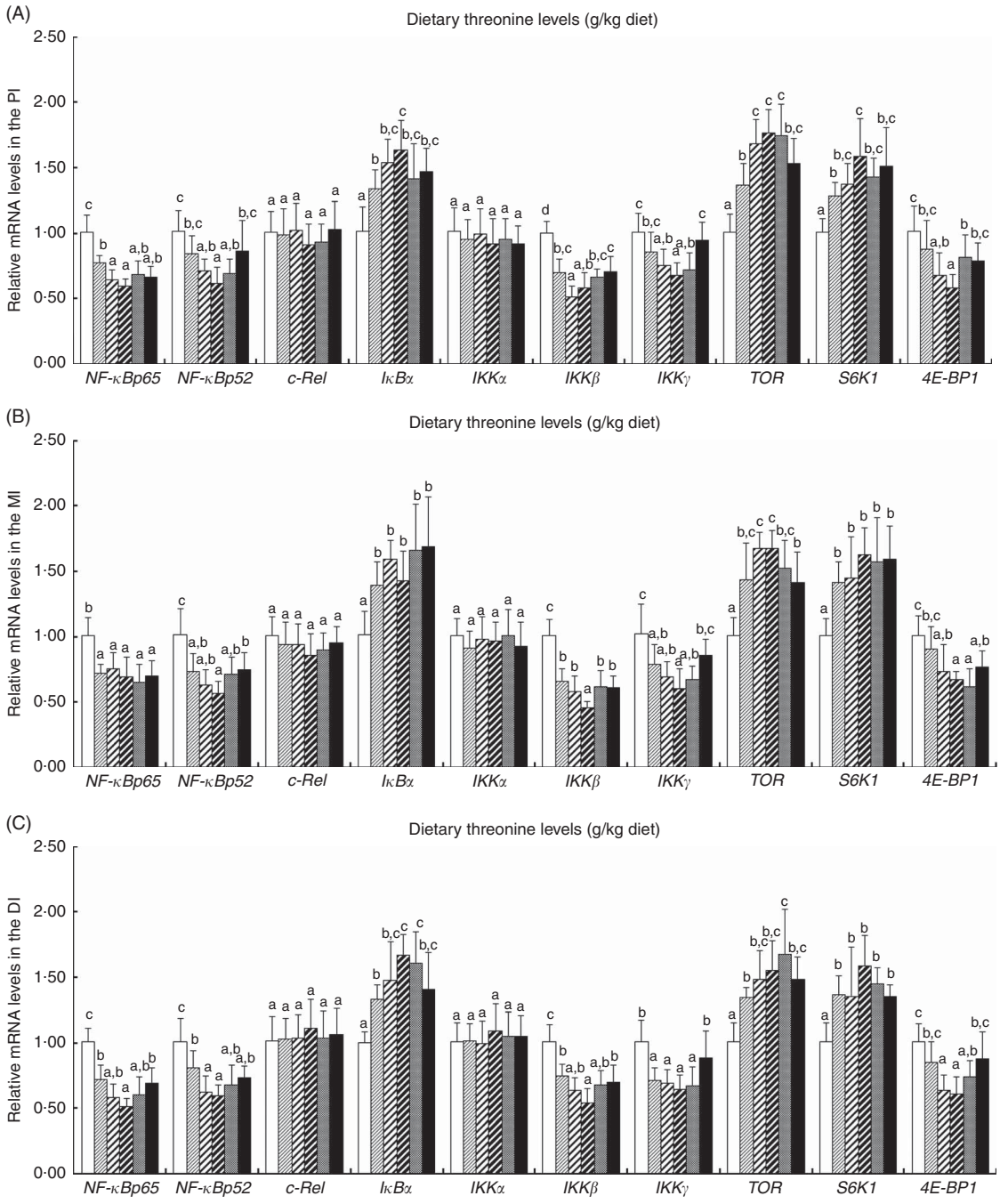

Fig. 5. Relative expression of *NF-κB p65*, *NF-κB p52*, *c-Rel*, *inhibitor of κBα* (*IκBα*), *IκB kinases* (*IKKα*, *IKKβ*, *IKKγ*), *target of rapamycin* (*TOR*), *S6 kinase* (*S6K1*) *and eIF4E-binding protein 1* (*4E-BP1*) in the proximal intestine (PI) (A), middle intestine (MI) (B) and distal intestine (DI) (C) of fish fed diets containing graded levels of threonine for 8 weeks after infection with *Aeromonas hydrophila* for 14 d. Values are means (six fishes per group), and standard deviations represented by vertical bars. □, Thr 3·99; ▨, Thr 7·70; ▨, Thr 10·72; ▨, Thr 14·10; ▨, Thr 17·96; ■, Thr 21·66. ^{a,b,c} Mean values with unlike letters are significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

and MI, IgM contents in MI and DI, ACP activities and contents of C3 and C4 in PI, MI and DI, mRNA levels of *β-defensin1*, *IgM* and *IgZ* in the MI and DI, *Hepcidin*, *LEAP-2A* and *LEAP-2B* in the three intestinal segments of juvenile grass carp after infection with *A. hydrophila*, indicating that threonine deficiency could depress the fish intestinal immunity.

Interestingly, the distinct effects of threonine deficiency on LA, IgM and *β-defensin1* was first observed in the different intestinal segments of juvenile grass carp. The possible reasons for the diverse results are considered in the next discussion. First, threonine deficiency decreased the LA in the PI and MI but increased it in the DI of juvenile grass carp, which might be related to *Cu/Zn-SOD* and nitric oxide. Habte-Tsion *et al.*[12] demonstrated that threonine deficiency could enhance the *Cu-Zn-SOD* mRNA level in the distal part but suppress it in the proximal part of intestines in the blunt snout bream. In mice

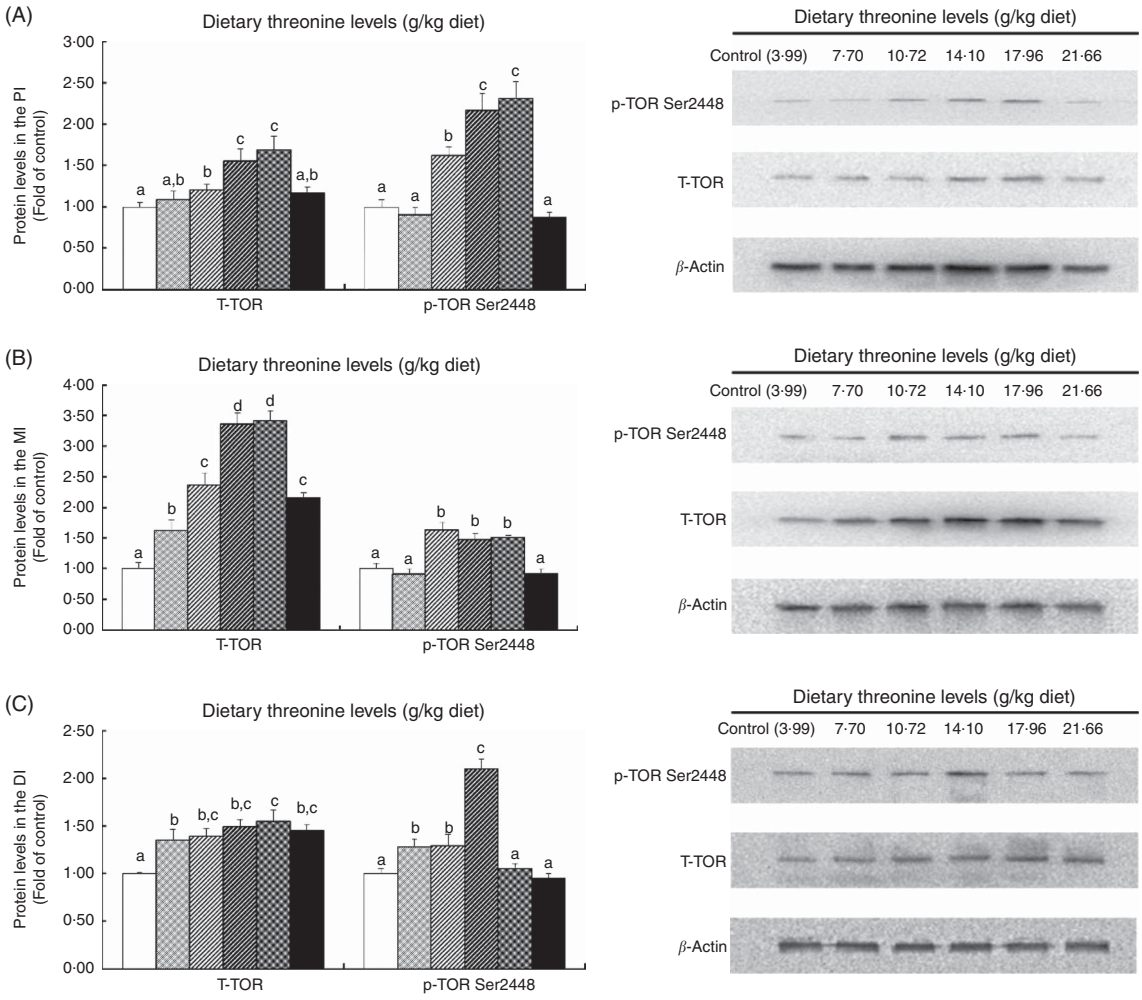

Fig. 6. Western blot analysis of total target of rapamycin (T-TOR) protein phosphorylation at Ser2448 (p-TOR Ser2448) in the proximal intestine (PI) (A), middle intestine (MI) (B) and distal intestine (DI) (C) of fish fed diets containing graded levels of threonine for 8 weeks after infection with *Aeromonas hydrophila* for 14 d. Values are means (three replicates per group), and standard deviations represented by vertical bars. □, Thr 3·99; ▨, Thr 7·70; ▨, Thr 10·72; ▨, Thr 14·10; ▩, Thr 17·96; ■, Thr 21·66. [a,b,c,d] Mean values with unlike letters were significantly different between treatments ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

renal, *Cu/Zn-SOD* deletion could decrease the nitric oxide level[72], which could hinder the release of LA in rabbit neutrophils[73]. Hence, we hypothesised that threonine deficiency down-regulated the *Cu/Zn-SOD* expression to decrease the nitric oxide level, thereby decreasing the LA in the PI and MI, while having adverse effects in the DI of fish. However, it warrants further investigation to support our hypothesis. Second, threonine deficiency down-regulated *IgM* mRNA levels and contents in the MI and DI (rather than PI) of juvenile grass carp, which might be related to the catabolite butyrate derived from threonine and different immunological relevance in the intestines *per se*. Smith *et al.*[74] confirmed that the production of butyrate decreased when threonine was insufficient in the distal parts of the intestine in humans. In bovine lymphocytes, insufficient butyrate decreased the synthesis of *IgM*[75]. Therefore, we suppose that threonine deficiency might decrease butyrate production to reduce IgM in the MI and DI (rather than PI) of fish, which needs further investigation. In the sea bass, a gradually increasing number of IgM^+ cells were established from anterior and middle to the posterior part of the intestines, which suggested a higher immunological relevance for the posterior gut, as the same finding as in the Atlantic halibut[76,77]. As we know, threonine could contribute to the proliferation of lymphocyte in the weaned pigs[10]. Thus, compared with the anterior part of the intestines, we hypothesised that threonine increased the IgM production only in the MI and DI of juvenile grass carp partly because of promoting larger amount of IgM^+ cells, which were distributed predominantly in the posterior parts of intestines. However, the hypothesis remains to be investigated further. Third, threonine deficiency down-regulated *β-defensin1* mRNA level in the MI and DI (not PI) of juvenile grass carp, which is likely to be relevant to *TGFβ*1. In this study, we observed that threonine deficiency down-regulated *TGFβ1* mRNA levels in the intestines of juvenile grass carp. Leppäranta *et al.*[78] reported that suppression of *TGFβ*1 could inhibit the transcription factor *GATA-6* expression in A549 cells. In mature mice, inhibited *GATA-6* was able to down-regulate the *Apoa1* mRNA levels in the posterior

but not in the anterior parts of the intestine[79]. In humans, it was reported that the absence of *Apoa1* decreased the *PGE2* production, which could down-regulate *β-defensin* mRNA levels[80,81]. Therefore, we presumed that threonine deficiency could decline *TGF-β1* mRNA levels to down-regulate *GATA-6* expression, thus decreasing *Apoa1* and *PGE2* mRNA levels only in the MI and DI (not PI) to decrease *β-defensin1* mRNA levels only in the MI and DI (not PI) of fish. However, the hypothesis needs further verification.

In addition, fish intestinal immune function is also associated with its inflammatory responses, which are primarily mediated by cytokines[21] and related signalling molecules[25,26]. Therefore, we next examined the effects of threonine on inflammatory cytokines and explored the possible mechanism by investigating the actions of threonine on *NF-κB*- and *TOR*-related signalling molecules in the intestines of juvenile grass carp after infection with *A. hydrophila*.

Threonine deficiency aggravated intestinal inflammation associated with NF-κB and target of rapamycin signalling pathways in fish after infection with Aeromonas hydrophila

Threonine deficiency triggered intestinal inflammation by up-regulating pro-inflammatory cytokines partly through the NF-κB signalling pathway in fish after infection with A. hydrophila. Generally, it is noteworthy that intestinal immune function can be activated by mediating pro-inflammatory cytokines such as *TNF-α* and *IL-1β*, the up-regulation of which can trigger intestinal inflammation in fish[19]. In this study, we systematically examined the effects of threonine on multiple pro-inflammatory cytokine responses in fish intestines after infection with *A. hydrophila*. Our study showed that, compared with optimal threonine supplementation, threonine deficiency up-regulated the pro-inflammatory cytokines *TNF-α*, *IL-1β*, *IFN-γ2*, *IL-6*, *IL-8* and *IL-17D* mRNA levels in three intestinal segments of juvenile grass carp after infection with *A. hydrophila*, suggesting that threonine deficiency aggravated the inflammatory responses in fish intestines. Furthermore, studies confirmed that cytokine production could be modulated by the signalling regulators of *NF-κB* in humans[25]. Therefore, we next investigated the effects of threonine on *NF-κB* signalling pathways in the intestines of juvenile grass carp after infection with *A. hydrophila*.

NF-κB is a transcription factor containing three subunits (*p65/p52/c-Rel*), which was traps activated by its inhibitor *IκBα* that could be degraded by IKK complex (including *IKKα*, *IKKβ* and *IKKγ*), and the activation of which could up-regulate pro-inflammatory cytokine (such as *IL-1β* and *IL-8*) expression in mammals[82]. In this study, compared with threonine supplementation, threonine deficiency induced the up-regulation of *NF-κB* (p65 and p52 (not c-Rel)), *IKK* (*β* and *γ* (not *α*)) and the down-regulation of *IκBα* mRNA levels in the three intestinal segments of juvenile grass carp after infection with *A. hydrophila*. Correlation analysis (Table 6) indicated that the mRNA levels of pro-inflammatory cytokines (*TNF-α*, *IFN-γ2*, *IL-6*, *IL-8* and *IL-17D*) were positively related to *NF-κBp65* or *NF-κBp52* in the three intestinal segments of fish and that the mRNA levels of *IKKβ* and *IKKγ* in the PI and DI were present the inverse correlation with *IκBα* which were negatively correlated with *NF-κBp65* and *NF-κBp52*, respectively. Meanwhile, the mRNA levels of *IKKβ* showed the adverse

Table 6. Correlation coefficients of genes relative expression in the intestine

		PI		MI		DI	
Dependent parameters	Independent parameters	Correlation coefficients	*P*	Correlation coefficients	*P*	Correlation coefficients	*P*
NF-κB p65	*TNF-α*	+0·912	<0·05	+0·848	<0·05	+0·956	<0·01
	IL-1β	+0·934	<0·01	–	–	+0·985	<0·01
	IFN-γ2	+0·962	<0·01	+0·876	<0·05	+0·942	<0·01
	IL-6	+0·947	<0·01	+0·842	<0·05	+0·950	<0·01
	IL-8	+0·986	<0·01	+0·746	=0·089	+0·961	<0·01
	IL-17D	+0·847	<0·05	+0·833	<0·05	+0·900	<0·05
NF-κB p52	*TNF-α*	+0·903	<0·05	+0·976	<0·01	+0·942	<0·01
	IL-1β	+0·870	<0·05	+0·926	<0·01	+0·986	<0·01
	IFN-γ2	+0·915	<0·05	+0·952	<0·01	+0·943	<0·01
	IL-6	+0·891	<0·05	+0·970	<0·01	+0·954	<0·01
	IL-8	+0·842	<0·05	+0·842	<0·05	+0·972	<0·01
	IL-17D	+0·920	<0·01	+0·900	<0·05	+0·891	<0·05
IκBα	*NF-κB p65*	−0·988	<0·01	−0·882	<0·05	−0·975	<0·01
	NF-κB p52	−0·893	<0·05	–	–	−0·951	<0·01
	IKKβ	−0·931	<0·01	−0·773	−0·071	−0·959	<0·01
	IKKγ	−0·775	=0·071	–	–	−0·872	<0·05
TOR	*TGF-β1*	+0·976	<0·01	+0·976	<0·01	+0·836	<0·05
	TGF-β2	+0·900	<0·05	+0·993	<0·01	+0·841	<0·05
	IL-4/13A	+0·980	<0·01	+0·971	<0·01	+0·860	<0·05
	IL-10	+0·987	<0·01	+0·917	<0·05	+0·888	<0·05
	S6K1	+0·900	<0·05	+0·850	<0·05	+0·895	<0·05
	4E-BP1	−0·896	<0·05	+0·820	<0·05	−0·767	=0·075

PI, proximal intestine; MI, mid intestine; DI, distal intestine; IKK, IκB kinase; TGF, transforming growth factor; S6K1, ribosomal protein S6 kinases 1; 4E-BP1, eIF4E-binding protein 1; TOR, target of rapamycin.

tendency to *IκBα*, which was negatively correlated to *NF-κBp65* in the MI of juvenile grass carp. The results given above implied that threonine deficiency up-regulated pro-inflammatory cytokine mRNA levels, which was partially attributed to *IKK* (*β* and *γ* (not *α*))/*IκBα*/*NF-κB* (p65 and p52 (not c-Rel)) signalling, thus triggering inflammation in fish.

Surprisingly, no significant differences were found in the *IL-12p35*, *IL-12p40*, *c-Rel* and *IKKα* mRNA levels of three intestinal segments for fish fed graded levels of threonine diets after infection with *A. hydrophila*. The potential reasons might be explained as follows. First, the fact that threonine deficiency did not affect the intestinal *IL-12* (*p35* and *p40*) mRNA levels might be related to the unchanged *c-Rel*. In mice, it was reported that c-Rel was essential for the activation of the *IL-12 p35* and *IL-12p40* expressions in dendritic cells and macrophages[83], respectively. However, our study displayed that threonine deficiency had no impact on *c-Rel* mRNA levels in the three intestinal segments of fish, supporting our hypothesis. As for the unchanged *c-Rel* mRNA levels by threonine, it might be related to glutamate. Hamard *et al.*[84] demonstrated that threonine deficiency could accumulate the glutamate levels in the plasma of pigs. A study has shown that glutamate had no impact on *c-Rel* binding activities in mice[85]. Thus, the fact that threonine deficiency did not alter the *c-Rel* transcript abundances might be partially related to the enhanced glutamate levels in fish intestines. However, the speculated reason still remains to be elucidated further. Second, the reason that threonine deficiency elevated *IKKβ* and *IKKγ* but not *IKKα* mRNA levels in the three intestinal segments of juvenile grass carp might be related to *IFN-γ* altering *PKCζ*. In this study, we observed that threonine deficiency up-regulated *IFN-γ* mRNA levels in the intestines of juvenile grass carp. In mice, it was found that *IFN-γ* could enhance the *PKCζ* levels[86], which could up-regulate *IKKβ* and *IKKγ* but ignore *IKKα* expression[87]. Hence, we hypothesised that threonine deficiency up-regulated the *IFN-γ* mRNA levels, partly resulting in increasing *PKCζ* levels, thus leading to the up-regulation of *IKKβ* and *IKKγ* (not *IKKα*) in fish intestines. However, our hypothesis still needs further investigation. In addition, except for up-regulation of pro-inflammatory cytokines, down-regulation of anti-inflammatory cytokines can also initiate the inflammation process[88]. Then, we next examined the effects of threonine on anti-inflammatory cytokines in the intestines of juvenile grass carp after infection with *A. hydrophila*.

Threonine deficiency induced intestinal inflammation via down-regulating the anti-inflammatory cytokines partly associated with target of rapamycin signalling pathway in fish after infection with Aeromonas hydrophila. In humans, it was confirmed that the down-regulation of anti-inflammatory cytokines (such as *TGF-β*, *IL-4/13* and *IL-10*) could aggravate the inflammation process[20], which could be regulated by *TOR* signalling via inhibiting ribosomal protein *S6K1* and activating *4E-BP1*[89]. In this study, for the first time, we probed the effects of threonine on anti-inflammatory cytokines in animal intestines after infection with *A. hydrophila*. Current results showed that, compared with optimal threonine supplementation, threonine deficiency down-regulated the anti-inflammatory cytokines *TGF-β1*, *TGF-β2*, *IL-4/13A* (not *IL-4/13B*) and *IL-10* transcript abundances in the PI, MI and DI of juvenile grass carp after infection with *A. hydrophila*, suggesting that threonine deficiency triggered the inflammatory responses via down-regulating anti-inflammatory cytokines in fish intestines. Furthermore, our data displayed that, compared with optimal threonine supplementation, threonine deficiency down-regulated *TOR* and *S6K1* mRNA levels, decreased *TOR* protein and its phosphorylation levels and up-regulated *4E-BP1* mRNA levels in the three intestinal segments of juvenile grass carp. Correlation analyses (Table 6) indicated that those anti-inflammatory cytokine mRNA levels (*TGF-β1*, *TGF-β2*, *IL-4/13A*, *IL-10* and *S6K1*) were positively related to TOR, which were negatively related to *4E-BP1* in the three intestinal segments of juvenile grass carp. The above observations manifested that threonine deficiency down-regulated anti-inflammatory cytokines partially because of the abridgement of the signal cascades ((*TOR*/(*S6K1* and *4E-BP1*)) in fish intestines.

Nevertheless, threonine deficiency down-regulated *IL-4/13A* (rather than *IL-4/13B*) mRNA levels in the three intestinal segments of juvenile grass carp, which might be explained by *TOR* and transcription factor *GATA-3*. In this study, threonine deficiency down-regulates *TOR* mRNA levels in the three intestinal segments of juvenile grass carp. It was confirmed that inhibition of *TOR* could down-regulate the *GATA-3* expression in mice $CD4^+$ T cells[90]. In Fugu, *GATA-3* could precede binding to presumable promoter region for sharing a motif of TATA box in *IL-4/13A* gene but not *IL-4/13B*[91]. Therefore, we speculated that threonine deficiency down-regulated *TOR* to diminish the *GATA-3* expression, thus leading to attenuating the binding to *IL-4/13A* (not *IL-4/13B*) to down-regulate the *IL-4/13A* (not *IL-4/13B*) mRNA levels in fish intestines. However, the possible supposition still remains to be elucidated.

Comparison of optimal threonine levels for juvenile grass carp based on different indices

In the context, threonine deficiency decreased growth performance, exaggerated enteritis morbidity, attenuated intestinal immunity and impaired intestinal inflammation response in juvenile grass carp after infection with *A. hydrophila*. On the basis of SGR, the threonine requirement was estimated to be 14·53 g threonine/kg diet (4·48 g threonine/100 g protein), which is a little higher than that recommended by Gao *et al.*[37] with 13·7 g threonine/kg diet (3·61 g threonine/100 g protein). To our knowledge, the higher the growth rate, the more adequate nutrients should be required in fish[92]. In this study, fish fed optimal threonine level showed a slightly higher SGR than that described by Gao *et al.*[37]. Besides, based on the protecting fish against enteritis morbidity and IgM content, threonine requirements were estimated to be 15·05 g threonine/kg diet (4·64 g threonine/100 g protein) and 15·17 g threonine/kg diet (4·68 g threonine/100 g protein), respectively (Fig. 7 and 8). Comparatively, the requirements for against enteritis morbidity

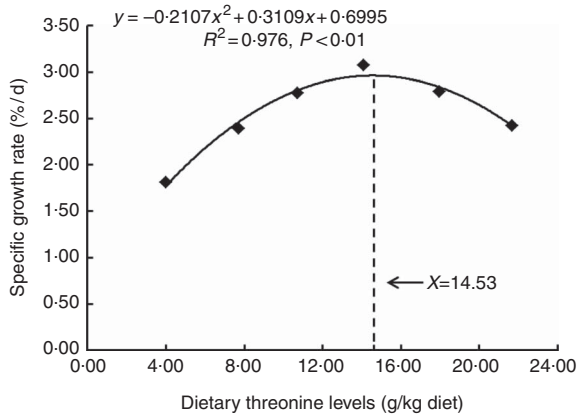

Fig. 7. Quadratic regression analysis of specific growth rate for the fish fed diets containing graded threonine levels for 8 weeks.

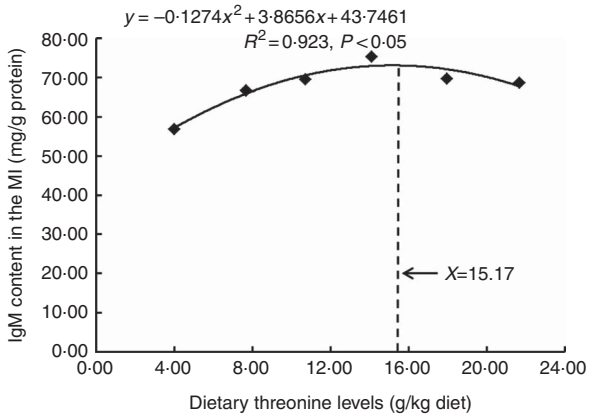

Fig. 8. Quadratic regression analysis of IgM for the fish fed diets containing graded threonine levels for 8 weeks after infection with *Aeromonas hydrophila* for 14 d. MI, middle intestine.

and improving immune index were close to or slightly higher than those for the growth performance, suggesting that a little more threonine supplementation is required for assuring intestinal health of fish.

Conclusions

In summary (Fig. 9), on the basis of the previous study about threonine on the growth performance of juvenile grass carp, for the first time, we systematically demonstrated that dietary threonine deficiency depressed the intestine immune function in fish by regulating immune-related signalling molecules after infection with *A. hydrophila*, as displayed in the following aspects: (1) threonine deficiency decreased the resistance against enteritis and attenuated intestinal immunity by reducing innate and adaptive immune components including LA (not in DI), ACP, IgM (not in PI), C3, C4 and antimicrobial peptide transcript abundances including *hepcidin*, *LEAP-2A*, *LEAP-2B*, *IgZ*, *IgM* and *β-defensin1* (not in PI); (2) threonine deficiency aggravated intestinal inflammation response by up-regulating pro-inflammatory cytokine *TNF-α*, *IL-1β*, *IFN-γ2*, *IL-6*, *IL-8* and *IL-17D* (not *IL-12p35* and *IL-12p40*) gene expression partly associated with NF-κB signalling pathway ((*IKKβ*, *IKKγ* but not *IKKα*)/*IκBα*/(*NF-κB p65*, *NF-κB p52* but not *c-Rel*)) and anti-inflammatory cytokines *TGF-β1*, *TGF-β2*, *IL-4/13A* (not *IL-4/13B*) and *IL-10* mRNA transcript levels partly through *TOR* signalling (*TOR*/(*S6K1* and *4E-BP1*)). In addition, based on quadratic regression for SGR, protecting fish against the enteritis morbidity and IgM content, dietary threonine requirements for juvenile grass carp were estimated to be 14·53 g threonine/kg diet (4·48 g threonine/100 g protein), 15·05 g threonine/kg diet (4·64 g threonine/100 g protein) and 15·17 g threonine/kg diet (4·68 g threonine/100 g protein), respectively.

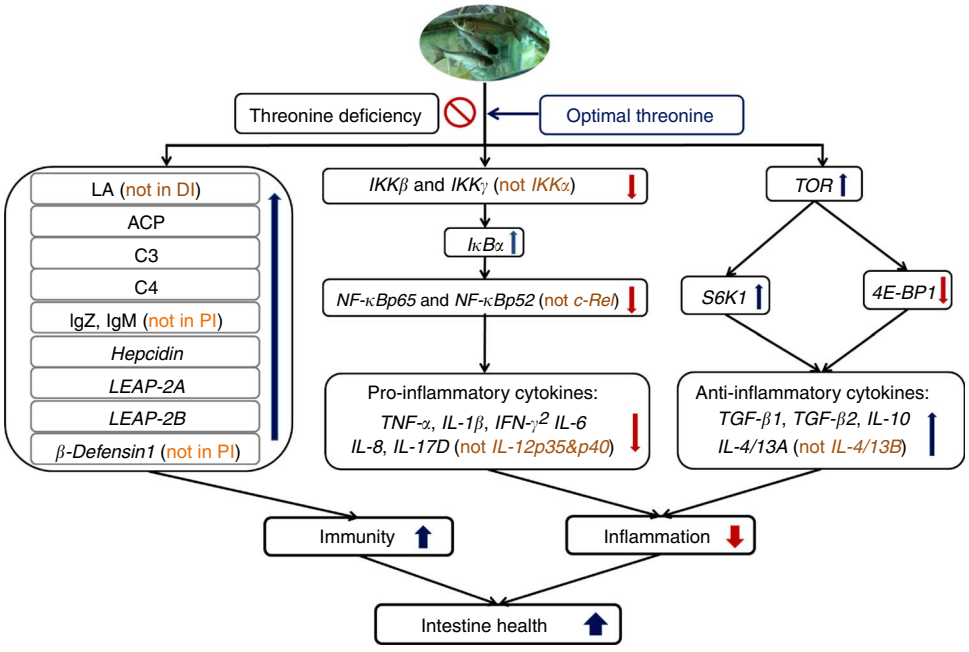

Fig. 9. Potential action pathways of dietary threonine regulating intestinal immunity and inflammation response of fish. LA, lysozyme activities; DI, distal intestine; IKK, IκB kinase; TOR, target of rapamycin; ACP, acid phosphatase; C3 and C4, complements; PI, proximal intestine; S6K1, ribosomal protein S6 kinases 1; 4E-BP1, eIF4E-binding protein 1; LEAP, liver-expressed antimicrobial peptide; IFN-γ, interferon γ; TGF, transforming growth factor.

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The author's contributions are as follows: X.-Q. Z. and L. F. designed the study; Y.-W. D., W.-D. J. and L. F. conducted the study and analysed the data; Y. L., P. W., J. J., S.-Y. K., L. T., W.-N.T. and Y.-A. Z. participated in the interpretation of the results; Y.-W. D. and W.-D. J. wrote the manuscript; X.-Q. Z. had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

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