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Supplementation exogenous bile acid improved growth and intestinal immune function associated with NF-κB and TOR signalling pathways in on-growing grass carp (*Ctenopharyngodon idella*): Enhancement the effect of protein-sparing by dietary lipid

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ABSTRACT

This study investigated the effects of bile acid (BA) supplementation on growth performance, intestinal immune function and the mRNA expression of the related signalling molecules in on-growing grass carp (*Ctenopharyngodon idella*). A total of 540 healthy grass carp (mean weight 179.85 ± 1.34 g) were fed a normal protein and lipid (NPNL) diet containing 29% crude protein (CP) and 5% ether extract (EE), and five low-protein and high-lipid (LPHL) diets (26% CP, 6% EE) with graded levels of BA (0–320 mg/kg diet) for 50 days. The fish were then challenged with *Aeromonas hydrophila* for 14 days. The results indicated that compared with the NPNL diet, the LPHL diet (unsupplemented BA) suppressed the growth performance, intestinal development and enteritis resistance capability and impaired the partial intestinal immune function of on-growing grass carp. Whereas in the LPHL diet, optimal BA supplementation significantly improved fish growth performance (percent weight gain, specific growth rate, feed intake and feed efficiency) and intestinal growth and function (intestine weight, intestine length and intestosomatic index), increased beneficial bacteria *Lactobacillus* and *Bifidobacterium* amounts, decreased harmful bacteria *Aeromonas* and *Escherichia coli* amounts, elevated lysozyme and acid phosphatase activities, increased complement (C3 and C4) and immunoglobulin M contents, and upregulated β-defensin-1, hepcidin, liver expressed antimicrobial peptide 2A (LEAP-2A), LEAP-2B, Mucin2, interleukin 10 (IL-10), IL-11, transforming growth factor (TGF)-β1, TGF-β2, IL-4/13A (not IL-4/13B), TOR, S6K1 and inhibitor of κBα (IκBα) mRNA levels. In addition, optimal BA supplementation in the LPHL diet downregulated tumour necrosis factor α (TNF-α), interferon γ2 (IFN-γ2), IL-1β, IL-6, IL-8, IL-15, IL-17D, IL-12p35, IL-12p40 (rather than proximal intestine (PI) or mid intestine (MI), nuclear factor kappa B p65 (NF-κB p65) (except NF-κB p52), c-Rel, IκB kinase β (IKKβ), IKKγ (except IKKα), eIF4E-binding proteins (4E-BP)1 and 4E-BP2 mRNA levels in all three intestinal segments of on-growing grass carp ($P < 0.05$). These findings suggest that BA supplementation in the LPHL diet improves growth and intestinal immune function of fish. Furthermore, 240 mg/kg BA supplementation in the LPHL diet was superior to the NPNL diet in improving growth and enhancing intestinal immune function of fish. Finally, based on percent weight gain, feed intake, protecting fish against enteritis, lysozyme activity in MI and acid phosphatase activity in distal intestine (DI), the optimal BA supplementation for on-growing grass carp were estimated to be 168.98, 170.23, 166.67, 176.50 and 191.97 mg/kg diet, respectively.

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1. Introduction

In recent years, accumulated evidence points to the shortage of protein resources, with the rapid development of aquaculture [1,2]. Use of low-protein and high-lipid diets seems to offer the fish-farming industry a means of reducing protein consumption. A Study reported that a reduction of 2% crude protein and an increase of 1% crude lipid in the diet is better than using a normal lipid and normal protein level for growth performance and intestinal immune function in grass carp (*Ctenopharyngodon idella*) [3]. However, excess levels of lipid reduced the growth performance of grass carp and Chinese perch (*Siniperca chuatsi*) [4,5] and impaired the intestinal immune function of grass carp [6]. These negative effects might be partly because a high level of lipid cannot be digested completely in the intestine of the animal [7]. Therefore, it is very important to enhance the digestion of lipid. Bile acid (BA) are a diverse class of cholesterol-derived, amphipathic molecules that function as detergents to facilitate digestion and absorption of dietary lipid [8], which could be used as a feed additive in fish [9,10] and terrestrial animals [11]. A study found that dietary BA supplementation in a high lipid level diet could increase the growth of juvenile grass carp [7]. In fish, growth performance is strongly correlated with intestinal health, which partly relies on the intestinal immune barrier [12]. However, to date, no study has investigated the effects of BA on the intestinal immune barriers in fish. Adhami et al. [9] reported that supplemental BA could improve lipase activity of rainbow trout (*Oncorhynchus mykiss*). Our laboratory's previous study indicated that exogenous lipase supplementation could enhance the intestinal immune barriers of grass carp [3]. These data suggest a possible correlation between dietary BA and fish intestinal immune barriers, which is worthy of investigation.

In fish, the immune function of the intestine is highly correlated with the presence and activity of antibacterial compounds and immunoglobulins [13,14]. Meanwhile, cytokines, including pro-inflammatory cytokines and anti-inflammatory cytokines, also play an important role in the immune functions of the fish intestine [15]. Several studies have reported that cytokines are regulated by the transcription factors nuclear factor-κB (NF-κB) in disk abalone (*Haliotis discus discus*) [16] and target of rapamycin (TOR) in grass carp [17]. However, no reports at present have investigated the role of dietary BA on the antibacterial compounds, cytokines and related signalling pathway in fish. It has been reported that BA improve mouse liver phospholipid levels [18], which can enhance AcP activity and C3 content in the intestine of juvenile grass carp [19]. In the murine enteroendocrine cell line STC-1, BA could promote glucagon-like peptide-1 (GLP-1) secretion [20]. One study showed that GLP-1 could down-regulate cytokine IFN-γ production in human invariant natural killer T cells [21]. A study in Kupffer cells of Wistar rats reported that BA could activate the G-protein coupled receptor (GPCR) TGR5 [22], which could reduce NF-κB expression in human HepG2 cells [23]. In addition, in the biliary system of dogs, BA promotes cholesterol secretion [24], which could activate TOR signalling in the head kidney of grass carp [25]. These observations revealed that there may be a relationship between dietary BA and the intestinal immune barrier function, as well as the related signalling pathway in fish, which needs to be investigated further.

Grass carp is one of the most important freshwater fish species in the world and has important economic value [26]. The growth process of fish can be divided into different stages such as juvenile, on-growing and adult [27]. To our knowledge, the optimal level of feed additive in fish might vary with different growth stages and different indices [28]. The optimal level of taurine, which is based on weight gain for the juvenile stage turbot (*Scophthalmus maximus* L.) (6.3 ± 0.01 g), is higher (10 g/kg) than that of the on-growing stage turbot (165.9 ± 5.01 g) (5 g/kg) [29]. Additionally, a study from our

Table 1
Composition and nutrients contents of basal diet.

	Diet (%)				
	NPNL diet	LPHL diet		NPNL diet	LPHL diet
Ingredients			Nutrients content		
Fish meal	5.00	4.45	Crude protein[e]	29.05	26.04
Soybean meal	27.00	24.01	Crude lipid[e]	5.01	6.00
Cottonseed meal	14.56	12.95	n-3[f]	1.06	1.29
Rapeseed meal	16.79	14.93	n-6[f]	0.98	1.15
Fish oil	2.56	3.28	Available phosphorus[g]	0.40	0.40
Soybean meal	1.34	1.72			
Corn starch	0.00	4.70			
α-starch	26.85	26.85			
$Ca(H_2PO_4)_2$	1.35	1.35			
Vitamin premix[a]	1.00	1.00			
Mineral premix[b]	2.00	2.00			
Choline chloride premix [c]	1.00	1.00			
Ethoxyquin (30%)	0.05	0.05			
L-Trp (99.2%)	0.00	0.03			
Threonine (98.5%)	0.00	0.12			
DL-Met (99%)	0.50	0.56			
BA premix[d]	0.00	1.00			

[a] Per kilogram of vitamin premix (g/kg): retinyl acetate (500, 000 IU/g), 0.39; cholecalciferol (500, 000 IU/g), 0.20; D, L-α-tocopherol acetate (50%), 23.23; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.10; ascorhyl acetate (95%), 9.77; niacin (99%), 4.04; meso-inositol (98%), 28.23; calcium-D-pantothenate (98%), 3.85; riboflavin (80%), 0.73; pyridoxine hydrochloride (98%), 0.45. All ingredients were diluted with corn starch to 1 kg.

[b] Per kilogram of mineral premix (g/kg): $MnSO_4 \cdot H_2O$ (31.8% Mn), 2.66; $MgSO_4 \cdot H_2O$ (15.0% Mg), 200.00; $FeSO_4 \cdot H_2O$ (30.0% Fe), 12.25; $ZnSO_4 \cdot H_2O$ (34.5% Zn), 8.25; $CuSO_4 \cdot 5H_2O$ (25.0% Cu), 0.96; KI (74.9% I), 0.067; Na_2SeO_3 (44.7% Se), 0.0168; All ingredients were diluted with corn starch to 1 kg.

[c] Choline chloride premix (g/kg premix): Choline chloride premix (50%), 261.90 g; All ingredients were diluted with corn starch to 1 kg.

[d] Per kilogram of BA premix (g/kg): premix was added to obtain graded levels of BA, and the amount of corn starch was reduced to compensate according to Yang et al.[102].

[e] Crude protein and crude lipid contents were measured value.

[f] n-3 and n-6 contents were referenced to Zeng et al. [103], and calculated according to NRC (2011).

[g] Available phosphorus content was referenced to Liang et al. [104], and calculated according to NRC (2011).

laboratory reported that the optimal level of sodium butyrate based on protecting fish against enteritis morbidity (339.9 mg/kg) was higher than that based on percent weight gain (160.8 mg/kg) in grass carp (256–781 g). Until now, the optimal supplementation of BA for grass carp was only evaluated in the juvenile stage, which is based on percent weight gain (PWG) [30]. Hence, it is necessary to investigate the optimal BA supplementation of on-growing grass carp based on growth and other indices.

In this study, we systematically investigated the effects of BA on antimicrobial compounds, cytokines and the possible signalling of NF-κB and TOR. Understanding these effects may reveal the impacts of BA on immune function and the underlying mechanisms in the intestine of fish. In addition, on the premise of an LPHL diet, the optimal levels of BA supplementation based on different indices of on-growing grass carp were also estimated, which might provide a reference for the commercial feed formulation of on-growing grass carp.

2. Materials and methods

2.1. Experimental diet preparation

The formulation and proximate composition analysis of the diets are presented in Table 1. Fish meal, soybean meal, cottonseed meal and rapeseed meal were used as the dietary protein sources. Fish oil and soybean oil were used as the main dietary lipid sources. In the normal protein and lipid (NPNL) diet, the dietary protein and lipid levels were fixed at 29% and 5% as described by Xu et al. [31] and Ni et al. [4], respectively. In the low-protein and high-lipid (LPHL) diets, the dietary protein and lipid levels were confirmed at 26% and 6%, respectively, according to Ng et al. [32]. BA (Cool Chemical Technology (Beijing) Co., Ltd., extracted from pig bile, sodium cholate, $C_{24}H_{39}NaO_5$, purity 98.2%) were added to the LPHL diets to provide graded concentrations of 0 (unsupplemented), 80, 160, 240 and 320 mg/kg diet, respectively. After being prepared completely, the diets were stored at −20 °C until feeding, as described by Zhou et al. [7].

2.2. Growth trial and sample collection

All protocols were approved by the University of Sichuan Agricultural Animal Care Advisory Committee, Sichuan, China under permit No. PXR-S20163729. The grass carp obtained from fishers (Sichuan, China) were acclimated to the experimental environment for 4 weeks as described by Wen et al. [33]. Then, 540 fish (mean weight 179.85 ± 1.34 g) were randomly assigned to 18 experimental cages (1.4 length × 1.4 width × 1.4 height in metres), and the 18 experimental cages were divided into 6 treatments with three replicates each (at a density of 30 fish/cage). We placed the three replicates in three individual outdoor freshwater ponds, resulting in 6 cages (treatments) per pond. The size of the pond was 12 m in length, 9 m in width, and 1.8 m in height. Aeration of water occurred during the trial. A disc with a 100-cm diameter was placed in the bottom of each cage to collect the uneaten feed, according to Tang et al. [34]. In the feeding trial, fish were fed their respective diets (three replicates per diet) to apparent satiation, four times per day for 50 days. After feeding for 30 min, uneaten feed was collected, dried and weighed to calculate the feed intake (FI) as described by Tian et al. [35]. During the experimental period, dissolved oxygen was above 6.0 mg/L. The pH value and water temperature were measured to be 7.5 ± 0.3 and 20.73 ± 3.86 °C, respectively. The feeding trials were conducted under natural light and dark cycles, which was similar to Chen et al. [19].

Fish in each cage were weighed and counted at the initiation and termination of the feeding trial to calculate the growth performance related indices. After the growth trial, nine fish from each treatment were anaesthetised in a benzocaine bath as described by Geraldo et al. [36]. Then, the intestines of the fish were quickly separated, measured and weighted for calculating the index of intestine. The gall bladders of three fish per diet were collected for observing the morphology. The livers from three fish per diet were washed with physiological saline and preserved in a 4% paraformaldehyde solution for histological examination according to Wang et al. [37]. The intestinal contents of six fish from each group were collected for measuring the counts of *Aeromonas*, *Escherichia coli (E. coli), Bifidobacterium* and *Lactobacillus* by the method described by Spanggaard et al. [38].

2.3. Challenge trial and sample collection

After the growth trial, a challenge trial was conducted to study the influence of dietary BA supplementation on the intestinal immune function of on-growing grass carp; the challenge trial was performed in a similar manner to that described by Xu et al. [31]. Twenty-four fish from each treatment group were randomly collected with similar body weights and moved to labelled cages as described by Ng et al. [39], and these fish were acclimatized to the experimental condition for 5 days according to Xu et al. [31]. *A. hydrophila* was graciously supplied by the Veterinary Medicine College, Sichuan Agricultural University in China. After the acclimatization, each fish was challenged with an intraperitoneal injection of 1.0 ml of 2.5 × 10^8 colony-forming units (cfu) ml^{-1} *A. hydrophila*. The injected number of bacteria was a nonlethal dose that could effectively induce inflammation and consequently allow for the investigation on fish reactivity against a threatening disease according to our preliminary study data (unpublished data). The challenge test was conducted for 14 days according to Xu et al. [31] and our preliminary test. During the challenge trial, the experimental conditions and managements were the same as the feeding trial described by Pan et al. [40].

At the end of the challenge trial, all fish (twenty-four fish per diet) were fasted for 24 h [41,42]. After fasting, all fish were anaesthetised in a benzocaine bath as described by Geraylou et al. [36]. Then, all fish were sacrificed, the intestines was quickly removed, and scoring was performed to evaluate the severity of fish enteritis based on the method of Song et al. [43]. After that, the intestines were quickly segmented into the proximal intestine (PI), the mid intestine (MI) and the distal intestine (DI) and washed with physiological saline, after which the PI, MI and DI of twenty-one fish per diet were frozen in liquid nitrogen and stored at −80 °C for later analysis as described by Xu et al. [44]. The PI, MI and DI from three fish per diet were preserved in 4% paraformaldehyde solution for histological examination according to Wang et al. [37].

2.4. Immunological analysis

Immunological analysis was performed after the fish were challenged with *A. hydrophila*. Six intestinal samples per treatment were homogenized in 10 vol (w/v) ice-cold physiological saline and centrifuged at 6000 *g* for 20 min at 4 °C, and the supernatants were used for biochemical analysis as described by our previous laboratory study [45]. Lysozyme (LZ) activity was determined by a lysozyme kit (Jiancheng Bioengineering Institute, Nanjing, China), which measures the decrease in turbidity after the lysis of Micrococcus peptidoglycan in the cell wall, according to the procedures reported by Zhou et al. (2012) [46]. Acid phosphatase (AcP) activity was assayed with a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the procedures reported by Zhao et al. (2016) [47], briefly, after incubation for 30 min; then, the concentration of phenol was spectrophotometrically detected at 520 nm. Complements C3 and C4 were determined by using the immunoturbidimetry kit according to the method of Yin et al. (2009) [48]. Immunoglobulin M (IgM) content was measured by immunoturbidimetry kit (Zhejiang Elikan Biological Technology Co., Ltd., Zhejiang, China), according to the method of Takemura et al. (2016) [49].

2.5. Histological examination

The PI, MI and DI from three fish per diet (after challenged with *A. hydrophila*) were fixed in 4% paraformaldehyde solution, dehydrated in ethanol/methanol and embedded in paraffin according to Wang et al. [37]. The tissue was sectioned at 4 μm. The sections were stained using standard hematoxylin and eosin (H & E) and examined by a light microscope (Nikon Eclipse TS100, Nikon Corporation, Tokyo, Japan) according to the method of Kokou et al. [50].

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

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were performed according to our previous study [28]. Total RNA was extracted from the PI, MI and DI from six fish per diet (after challenged with *A. hydrophila*) using an RNAiso Plus Kit (TaKaRa, Dalian, China). Then, the quality and quantity of RNA were assessed using agarose gel (1%) electrophoresis and spectrophotometric analysis, respectively, as described by Luo et al. [45] and Betancor et al. [51]. Subsequently, the RNA was reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa) according to the manufacturer's instructions. PCR specific primers were designed according to the sequences that were cloned and published in the gene bank of grass carp for quantitative real-time PCR (Supplemental Table S1). 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. Target and housekeeping gene amplification efficiencies were calculated according to the specific gene standard curves generated from 10-fold serial dilutions. Gene transcription level was normalized to the β-actin content in each sample using the $2^{-\Delta\Delta CT}$ method [52,53].

2.7. Western blot analysis

The procedures for intestinal protein extract preparation, antibodies were used and western blotting was performed as in our previous study [54]. Briefly, the protein concentrations were determined by a bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology Inc., China). Protein samples (40 μg protein per lane) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane for Western blot analysis. After transfer, the membrane was blocked for 1.5 h with 0.5% BSA at room temperature and then incubated with a primary antibody overnight at 4 °C. We used the same anti-total TOR (AF6308, 1:1000 dilution), p-TOR Ser 2448 (AF3308, 1:1000 dilution), NF-κB p65 (AF5006, 1:750 dilution), Lamin B1 (AF5161, 1:1000 dilution) and β-Actin (AF7018, 1:3000 dilution) antibodies (Affinity BioReagents, Golden, Colorado, USA) as those in our previous study [54]. β-actin and Lamin B1 were used as control proteins for total and nuclear protein content, respectively. The blots were washed three times and followed by a 1.5 h incubation with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (A0208, 1:8000 dilution, Beyotime Biotechnology Inc., China) in tris-buffered saline with 0.1% Tween-20 (TBST). The immune complexes were visualised using electrochemiluminescence (ECL) reagents (Affinity Biosciences Inc., America). The Western blot bands were quantified using NIH Image 1.63 software (National Institutes of Mental Health, Bethesda, USA). Different treatments were expressed as fold change versus the NPNL group. This experiment was repeated for 3 fish/diet, and similar results were obtained each time.

2.8. Statistical analysis

The data of initial body weight (IBW), final body weight (FBW) and feed intake (FI) were used to calculate the percent weight gain (PWG), specific growth rate (SGR) and feed efficiency (FE) according to Siddik et al. [55] and Ai et al. [56]. The intestinal length (IL) and intestinal weight (IW) were used to calculate intestinal length index (ILI) and intestinal somatic index (ISI) according to Jiang et al. [57].

PWG = 100 × [FBW (g/fish) – IBW (g/fish)]/IBW (g/fish);

SGR = 100 × ln [final body weight (g)/initial body weight (g)]/days;

FE = 100 × [FBW (g/fish) – IBW (g/fish)]/FI (g/fish);

ISI = 100 × [wet intestine weight (g)/wet body weight (g)];

ILI = 100 × [intestine length (cm)/total body length (cm)];

The results are represented with the means ± SD. Data were subjected to one-way ANOVA followed by the Duncan's multiple-range test to determine significant differences among six treatment groups using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant. Quadratic regression model was used to estimate the optimal level of dietary BA for on-growing grass carp.

3. Results

3.1. The effects of exogenous BA supplementation in the LPHL diet on fish growth, intestinal growth and function, and intestinal bacterial counts of on-growing grass carp

As shown in Table 2. Compared with the NPNL diet, the grass carp fed the LPHL diet had lower FBW, PWG, SGR, FI, IW, IL, ISI, ILI, *Lactobacillus* and *Bifidobacterium* amounts ($P < 0.05$). There were no notable differences in FE and the amounts of *Aeromonas* and *E. coli* between the NPNL diet and the LPHL diet ($P > 0.05$). The FBW, PWG, SGR, FI, IW, ISI, IL, ILI, and amounts of *Lactobacillus* and *Bifidobacterium* all significantly increased with the dietary BA level up to 160 mg/kg ($P < 0.05$) and then significantly decreased ($P < 0.05$). The FE was significantly increased with the dietary BA level up to 160–240 mg/kg and then significantly decreased ($P < 0.05$). The *E. coli* and *Aeromonas* amounts were significantly decreased as the dietary BA levels increased to 160 mg/kg ($P < 0.05$), and raised significantly ($P < 0.05$). With dietary BA level up to 80 mg/kg diet, the FBW, PWG, SGR, FI, IW, IL, ILI and *Lactobacillus* amounts were significantly higher than those of fish fed the NPNL diet ($P < 0.05$), the *Aeromonas* and *E. coli* amounts were significantly lower than those of fish fed the NPNL diet ($P < 0.05$), and the ISI and *Bifidobacterium* amounts were comparable to those of the fish fed the NPNL diet.

3.2. The effects of exogenous BA supplementation in the LPHL diet on liver histology and gall bladder morphology

As displayed in Fig. 1. In the present study, there was no significant difference between the gall bladder morphology of fish fed the NPNL diet versus the LPHL diet. However, the appearance of the gall bladders changed from a dark green to a light green in colour as the supplemented level of BA increased from zero to 240 mg/kg diet. Fish fed the diet with 320 mg/kg BA were found to have yellow coloured bile and a small amount of solid present in their gall bladders. The gall bladder capsule of these fish was extremely fragile and easy to rupture.

As displayed in Fig. 2. In fish fed the NPNL diet (Fig. 2A), the clear liver cells were arranged well. Liver cell swelling and vacuolar degeneration were observed in fish fed the LPHL diet (Fig. 2B). The cell morphology of fish fed the 160 mg/kg BA supplementation in the LPHL diet (Fig. 2C) was similar to that of fish fed the NPNL diet. The hepatocytes were disorganized, some hepatocyte nuclei were concentrated or collapsed, and focal necrosis, leucocyte infiltration and hepatic sinusoid hyperaemia, and nuclear migration were observed in fish fed the LPHL diet supplemented with 320 mg/kg BA.

Table 2
Growth performance, weight (g/fish) and index and intestinal bacterial counts of on-growing grass carp (*Ctenopharyngodon idella*) fed the diets with graded level of BA for 50 days.

	NPNL diet	LPHL diet				
	Bile acids in the diet (mg/kg diet)					
	0	0	80	160	240	320
IBW[a]	179.33 ± 1.15^{a}	179.11 ± 1.02^{a}	180.89 ± 1.02^{a}	179.33 ± 1.15^{a}	181.33 ± 1.76^{a}	179.11 ± 0.38 ^{a}
FBW[a]	419.11 ± 8.70^{b}	383.78 ± 6.34^{a}	468.22 ± 9.71^{c}	533.33 ± 19.06^{d}	488.97 ± 15.71^{c}	416.67 ± 3.06^{b}
PWG[a]	133.69 ± 3.55^{b}	114.28 ± 4.42^{a}	158.83 ± 4.00^{c}	197.38 ± 9.87^{e}	169.63 ± 7.07^{d}	132.63 ± 1.68^{b}
SGR[a]	1.70 ± 0.03^{b}	1.52 ± 0.04^{a}	1.90 ± 0.03^{c}	2.18 ± 0.07^{d}	1.98 ± 0.05^{c}	1.69 ± 0.01^{b}
FI[a]	468.28 ± 1.45^{c}	415.96 ± 1.23^{a}	505.21 ± 1.21^{d}	565.63 ± 0.52^{f}	511.22 ± 0.57^{e}	445.13 ± 1.62^{b}
FE[a]	51.20 ± 1.51^{ab}	49.21 ± 1.81^{a}	56.87 ± 1.66^{cd}	62.59 ± 3.33^{e}	60.18 ± 2.80^{de}	53.37 ± 0.87^{bc}
IW[b]	8.14 ± 0.49^{b}	7.09 ± 0.56^{a}	9.11 ± 0.78^{c}	12.20 ± 0.61^{e}	10.37 ± 0.80^{d}	7.97 ± 0.57^{b}
ISI[b]	1.90 ± 0.13b^{c}	1.69 ± 0.15^{a}	2.05 ± 0.09^{cd}	2.26 ± 0.15^{e}	2.10 ± 0.16^{d}	1.85 ± 0.14^{b}
IL[b]	48.79 ± 2.71^{b}	38.37 ± 3.88^{a}	55.64 ± 4.78^{c}	64.31 ± 4.17^{d}	58.33 ± 4.15^{c}	45.96 ± 3.58^{b}
ILI[b]	148.97 ± 8.18^{b}	117.45 ± 9.83^{a}	166.63 ± 10.60^{c}	180.35 ± 6.14^{d}	167.31 ± 14.49^{c}	138.35 ± 8.65^{b}
Aeromonas[c]	7.31 ± 0.17^{c}	7.43 ± 0.06^{cd}	7.03 ± 0.14^{b}	6.70 ± 0.17^{a}	7.08 ± 0.03^{b}	7.50 ± 0.02^{d}
E. coli[c]	7.09 ± 0.07^{c}	7.13 ± 0.03^{c}	6.88 ± 0.30^{b}	6.67 ± 0.18^{a}	6.90 ± 0.07^{b}	7.11 ± 0.05^{c}
Lactobacillus[c]	7.13 ± 0.02^{b}	7.05 ± 0.07^{a}	7.19 ± 0.06^{c}	7.28 ± 0.05^{d}	7.15 ± 0.01^{bc}	7.04 ± 0.02^{a}
Bifidobacterium[c]	7.22 ± 0.02^{b}	7.10 ± 0.04^{a}	7.23 ± 0.04^{b}	7.33 ± 0.08^{c}	7.23 ± 0.01^{b}	7.12 ± 0.02^{a}
Regression						
$Y_{FBW} = -0.0047x^2 + 1.6188x + 380.46$					$R^2 = 0.9693$	$P < 0.05$
$Y_{PWG} = -0.0026x^2 + 0.8787x + 112.28$					$R^2 = 0.9527$	$P < 0.05$
Y_{SGR} = -2E-05x^2 + 0.007x + 1.5135					$R^2 = 0.9696$	$P < 0.01$
$Y_{FI} = -0.0047x^2 + 1.6002x + 414.97$					$R^2 = 0.9611$	$P < 0.05$
$Y_{FE} = -0.0004x^2 + 0.1469x + 48.821$					$R^2 = 0.9824$	$P < 0.05$
$Y_{IW} = -0.0002x^2 + 0.0530x + 6.7755$					$R^2 = 0.8901$	$P = 0.11$
$Y_{IL} = -0.0008x^2 + 0.2864x + 38.388$					$R^2 = 0.9920$	$P < 0.01$

[a] Values are means ± SD for three replicate groups, with 30 fish in each group, and different superscripts in the same row are significantly different ($P < 0.05$). IBW: initial body weight (g/fish); FBW: final body weight (g/fish); PWG: percent weight gain (%); SGR: specific growth rate (%/day); FI: feed intake (g/fish); FE: feed efficiency (%).

[b] Values are means ± SD (n = 9), and different superscripts in the same row are significantly different ($P < 0.05$). IW: intestinal weight (g/fish), IL: intestinal length (cm/fish); ISI: intestinal somatic index (%); ILI: intestinal length index (%).

[c] Values are means ± SD (n = 6), and different superscripts in the same row are significantly different ($P < 0.05$). *Aeromonas* (log CFU/g intestine content); *E. coli, Escherichia coli* (log CFU/g intestine content); *Lactobacillus* (log CFU/g intestine content); *Bifidobacterium* (log CFU/g intestine content).

3.3. The effects of exogenous BA supplementation in the LPHL diet on the enteritis morbidity and intestinal histopathological lesions in on-growing grass carp after infection with A. hydrophila

As shown in Fig. 3. Compared with the NPNL diet, the enteritis morbidity in fish fed the LPHL diet was significantly greater ($P < 0.05$). BA supplementation in the LPHL diet significantly reduced the enteritis morbidity ($P < 0.05$), reaching a minimum when the BA level was up to 160 mg/kg diet, and then significantly increased the enteritis morbidity ($P < 0.05$). With dietary BA levels up to 80–240 mg/kg diet, the enteritis morbidity was significantly lower than in fish fed the NPNL diet ($P < 0.05$).

As shown in Fig. 4 and Fig. 5, compared with the NPNL diet and the 160 mg BA/kg diet, respectively, fish fed the LPHL diet showed noticeable red and swollen intestines. The histological results showed that the LPHL diet led to significant symptoms of blood capillary hyperaemia and lamina propria oedema after the on-growing grass carp were challenged with *A. hydrophila* in their intestines ($P < 0.05$).

3.4. The effects of exogenous BA supplementation in the LPHL diet on the immune parameters in all three intestinal segments of on-growing grass carp

As shown in Table 3. In the PI, compared with the NPNL diet, fish fed the LPHL diet showed lower activities of LZ and AcP, contents of IgM and C3 ($P < 0.05$). However, the C4 content had no notable difference between the 2 groups. The C3 content, LZ and AcP activities were increased significantly with the dietary BA level up to 160 mg/kg ($P < 0.05$), and then remarkably decreased ($P < 0.05$). IgM and C4

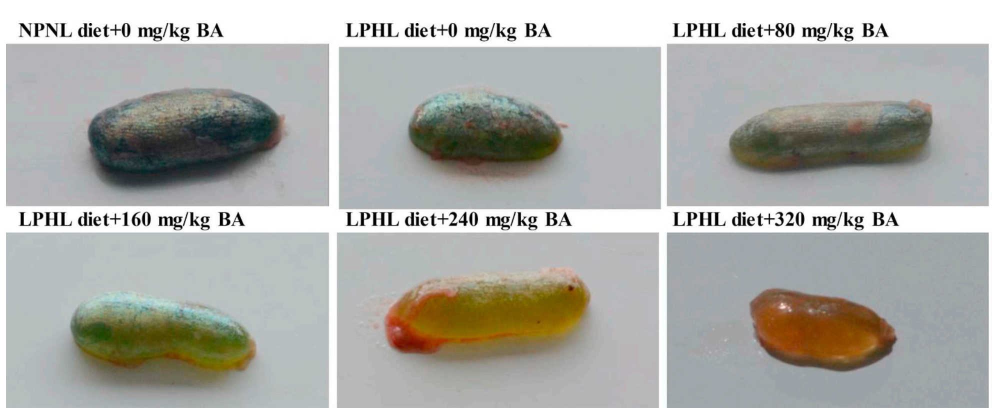

Fig. 1. Gall bladder morphology of on-growing grass carp fed with difference dietary BA supplementations. Note: The substance around the gall bladder was the remnant of the liver.

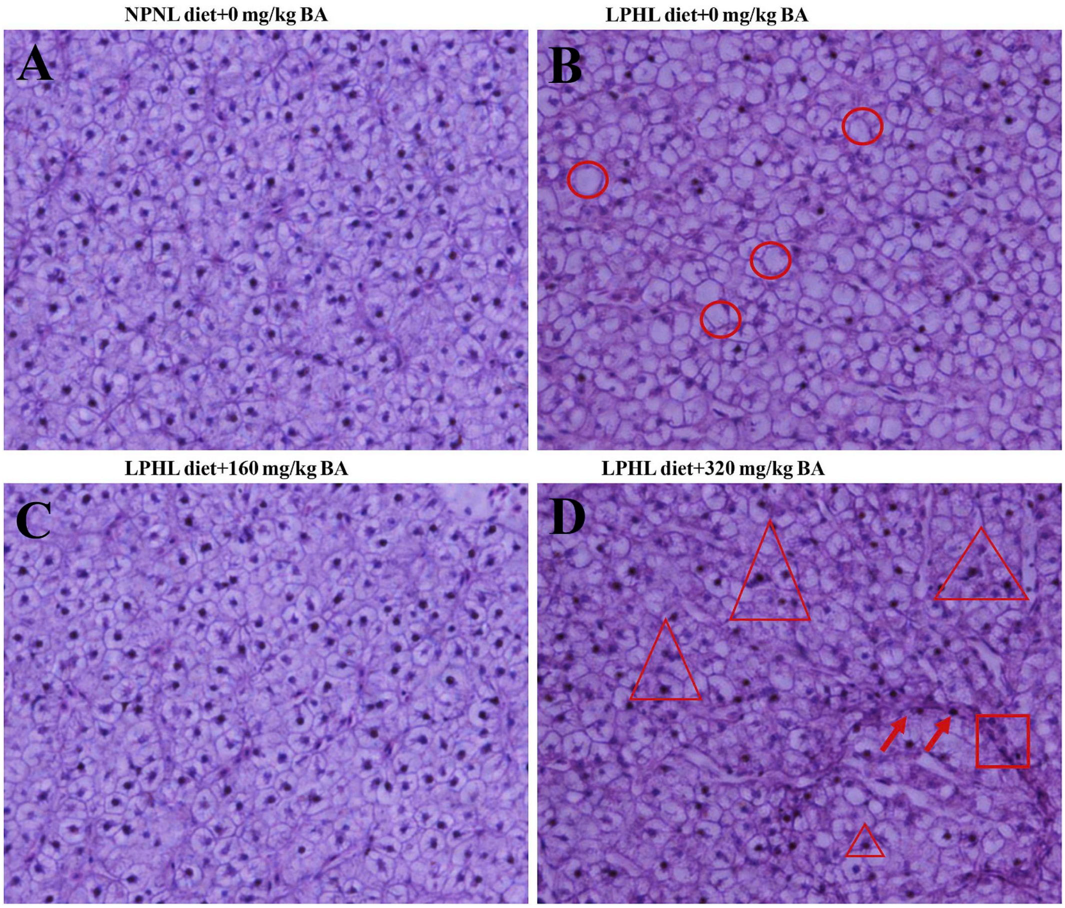

Fig. 2. Histologically observed liver of on-growing grass carp fed with difference dietary BA supplementations (Circle, liver cells swelling and vacuolar degeneration; Trilateral, hepatocytes disorganized, some hepatocyte nuclei concentrated, collapsed, and focal necrosis could be observed; Square, the leucocyte infiltration and hepatic sinusoid hyperemia; Arrowhead, nuclear migration; × 400; H & E (hematoxylin & eosin) staining. n = 3).

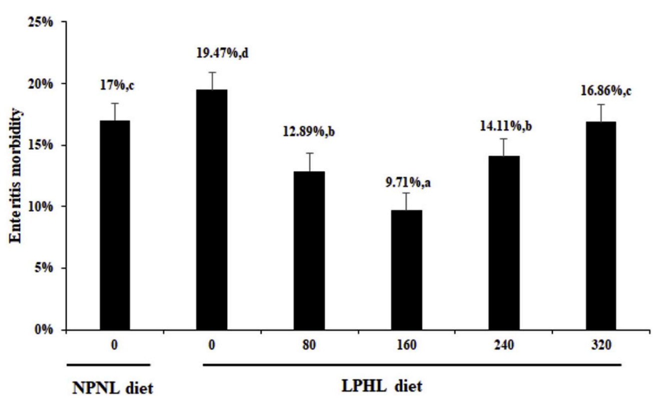

Fig. 3. Effects of dietary BA (mg/kg) supplementation in the LPHL diet on enteritis morbidity of on-growing grass carp after infection with *Aeromonas hydropila*. Values having different letters are significantly different ($P < 0.05$), n = 3 (8 fish per replicate).

levels were significantly and dose-dependently elevated with the increasing dietary BA levels; both IgM and C4 reached their peak levels with the 160 mg BA/kg diet, and then their levels gradually decreased. With dietary BA levels up to 80 mg/kg diet, the IgM content was comparable to those of fish fed the NPNL diet, the content of C3 and activities of AcP and LZ were significantly higher than those of fish fed the NPNL diet ($P < 0.05$). In the MI, compared with the NPNL diet, fish fed the LPHL diet showed lower activities of LZ and AcP, contents of C3 and C4 ($P < 0.05$). However, the IgM content had no notable difference between the 2 groups ($P > 0.05$). The C3 content, LZ and AcP activities were significantly increased as the BA levels increased to 160 mg/kg diet ($P < 0.05$), the contents of C4 and IgM were gradually raised as the dietary BA level up to 160 mg/kg ($P > 0.05$), and then all of them remarkably decreased ($P < 0.05$). With dietary BA levels up to 80 mg/kg diet, the C4 content was comparable to those of fish fed the NPNL diet, the content of C3, activities of AcP and LZ were significantly higher than those of fish fed the NPNL diet ($P < 0.05$). In the DI, compared with the NPNL diet, fish fed the LPHL diet had lower activities of LZ and AcP, contents of IgM and C4 ($P < 0.05$). However, the C3 content had no notable difference between the 2 groups ($P > 0.05$). The activities of AcP and LZ, contents of C3 and IgM were strikingly elevated as the BA levels increased to 160 or 240 mg/kg diet ($P < 0.05$), and then all of them remarkably decreased ($P < 0.05$). The C4 content was gradually upregulated with the increasing dietary BA levels up to 160 mg/kg and then gradually decreased. With dietary BA levels up to 80 mg/kg diet, the IgM content was comparable to those of fish fed the NPNL diet, the C4 content and activities of AcP and LZ were significantly higher than those of fish fed the NPNL diet ($P < 0.05$).

3.5. The effects of exogenous BA supplementation in the LPHL diet on the intestinal antimicrobial peptide mRNA levels of on-growing grass carp

As shown in Fig. 6. Compared with the NPNL diet, fish fed the LPHL diet had a lower β-defensin-1 mRNA level in the PI ($P < 0.05$). The mRNA levels of liver-expressed antimicrobial peptide 2A (LEAP-2A), LEAP-2B, hepcidin, Mucin 2 in all three intestinal segments and β-defensin-1 in the MI and DI had no notable difference between the 2

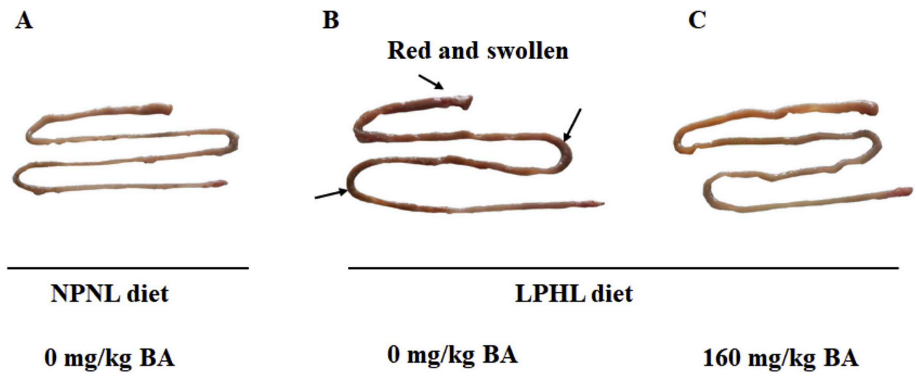

Fig. 4. The enteritis symptom of on-growing grass carp (*Ctenopharyngodon idella*) after infection of *A. hydrophila*. Compared with the LPHL diet, the NPNL diet and 160 mg/kg BA supplementation in the LPHL diet obviously alleviated enteritis symptom of on-growing grass carp [1].[1]Visibly red and swollen were observed for fish fed the LPHL diet after infection with *A. hydrophila*. A: NPNL diet; B: LPHL diet; C: 160 mg/kg BA supplementation in the LPHL diet. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

groups. The mRNA levels of β-defensin-1, LEAP-2A, LEAP-2B and Mucin2 in all three intestinal segments, as well as hepcidin in the PI and DI were gradually upregulated and hepcidin mRNA level in the MI were significantly raised as the dietary BA levels increased to 160 mg/kg diet ($P < 0.05$), then both of them were downregulated. With dietary BA level up to 80 mg/kg diet, the mRNA levels of β-defensin-1 in the PI was comparable to those of fish fed the NPNL diet ($P > 0.05$).

3.6. The effects of exogenous BA supplementation in the LPHL diet on the intestinal cytokines and related signalling molecules mRNA levels of on-growing grass carp

As shown in Fig. 7. In the PI, compared with the NPNL diet, fish fed the LPHL diet had higher mRNA levels of pro-inflammatory cytokines tumour necrosis factor α (TNF-α), interferon γ2 (IFN-γ2), interleukin 6 (IL-6), IL-15 and IL-17D ($P < 0.05$). The mRNA levels of IL-1β, IL-8, IL-12p35 and IL-12p40 had no notable difference between the 2 groups ($P > 0.05$). The mRNA levels of IL-1β, TNF-α, IFN-γ2, IL-6, IL-8 and IL-17D were all downregulated with dietary BA levels up to 160 mg/kg, and then upregulated. The mRNA levels of IL-12p35 and IL-15 were all downregulated with dietary BA levels up to 240 mg/kg, and then upregulated. Interestingly, the IL-12p40 mRNA level was not influenced by dietary BA levels ($P > 0.05$). With dietary BA levels up to 80 mg/kg diet, the mRNA level of IL-15 was comparable to those of the fish fed the NPNL diet, the mRNA levels of TNF-α, IFN-γ2, IL-6 and IL-17D were significantly lower than those of fish fed the NPNL diet ($P < 0.05$). In the MI, compared with the NPNL diet, fish fed the LPHL diet had higher mRNA levels of pro-inflammatory cytokines IL-1β, IL-6 and IL-17D ($P < 0.05$). The mRNA levels of TNF-α, IFN-γ2, IL-8, IL-12p35, IL-12p40 and IL-15 had no notable difference between the 2 groups ($P > 0.05$). The mRNA levels of IL-1β, TNF-α, IFN-γ2, IL-6, IL-8 and IL-17D were all downregulated with dietary BA levels up to 160 mg/kg, and then upregulated, The mRNA levels of IL-12p35 and IL-15 were all downregulated with dietary BA levels up to 240 mg/kg, and then

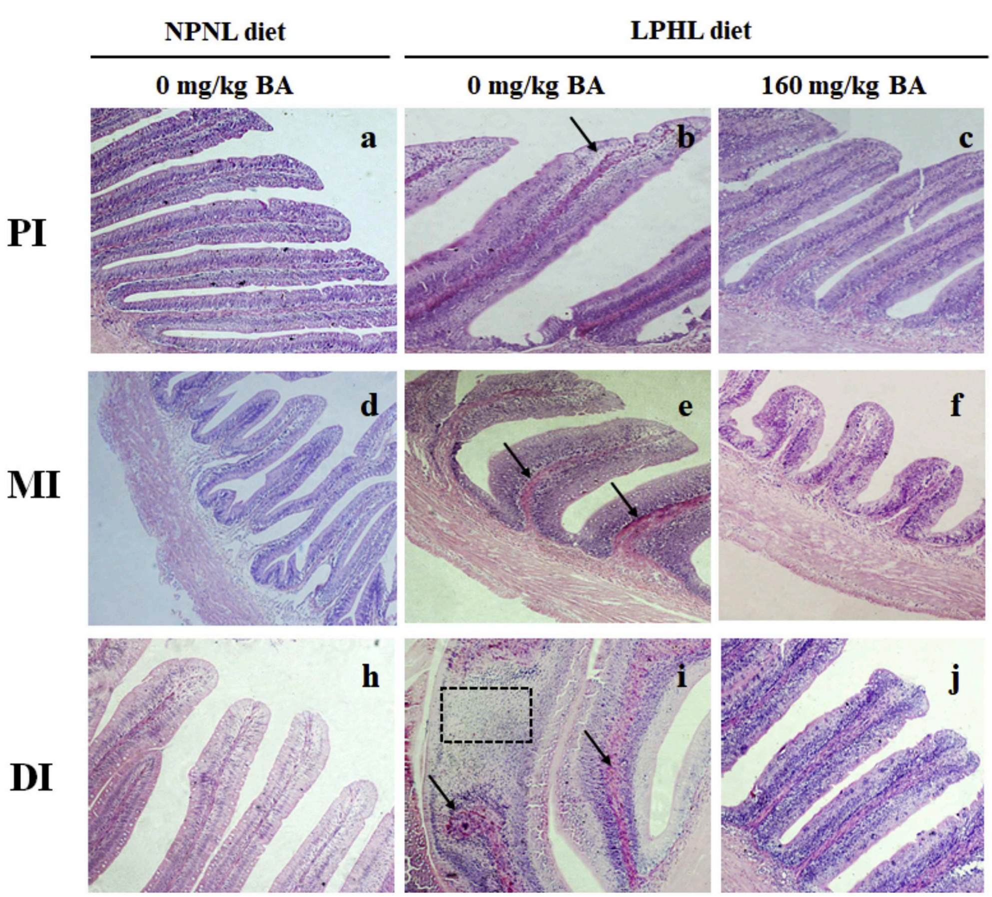

Fig. 5. Intestinal histological analysis of on-growing grass carp fed diets supplemented with BA after challenged with *A. hydrophila* for 14 days[1] [1]Box showed the oedema in the lamina propria. Arrowhead showed the blood capillary hyperemia. The NPNL diet (a, d, h); The LPHL diet (b, e, i); 160 mg/kg BA supplementation in the LPHL diet (c, f, j). The sections were H & E (hematoxylin & eosin) staining and observed at 100× original magnification, n = 3.

Table 3
Effects of dietary graded levels of BA on antimicrobial compounds in the PI, MI and DI of on-growing grass carp [a].

	NPNL diet	LPHL diet				
	Bile acid in the diet (mg/kg diet)					
	0	0	80	160	240	320
PI						
LZ	88.05 ± 7.87^{b}	56.56 ± 4.57^{a}	105.51 ± 9.58^{c}	138.48 ± 12.14^{d}	109.20 ± 10.44^{c}	85.50 ± 8.22^{b}
AcP	112.31 ± 10.72^{b}	71.18 ± 10.65^{a}	127.90 ± 7.25^{c}	171.25 ± 17.12^{d}	130.95 ± 10.37^{c}	103.43 ± 11.89^{b}
C3	22.35 ± 2.12^{b}	18.19 ± 1.72^{a}	24.90 ± 2.36^{c}	28.80 ± 2.33^{d}	25.12 ± 1.91^{c}	21.49 ± 2.11^{b}
C4	7.03 ± 0.52^{ab}	6.48 ± 0.72^{a}	7.47 ± 0.42^{bc}	8.05 ± 0.60^{c}	6.94 ± 0.30^{ab}	6.69 ± 0.52^{a}
IgM	47.81 ± 5.45^{b}	38.97 ± 3.82^{a}	49.57 ± 3.24^{b}	55.85 ± 5.96^{c}	51.46 ± 2.04^{bc}	46.32 ± 4.66^{b}
MI						
LZ	141.03 ± 15.29^{b}	113.12 ± 9.02^{a}	168.46 ± 14.87^{c}	198.96 ± 15.08^{d}	172.38 ± 14.07^{c}	144.32 ± 12.51^{b}
AcP	207.29 ± 15.33^{b}	175.55 ± 13.14^{a}	237.81 ± 17.07^{c}	286.57 ± 22.55^{d}	232.65 ± 20.32^{c}	195.01 ± 8.93^{ab}
C3	24.00 ± 1.69^{b}	19.25 ± 1.73^{a}	28.03 ± 2.67^{c}	32.13 ± 2.82^{d}	27.96 ± 2.94^{c}	23.85 ± 2.31^{b}
C4	11.74 ± 1.30^{b}	10.10 ± 1.68^{a}	12.88 ± 0.96^{bc}	13.84 ± 1.22^{c}	11.83 ± 0.43^{b}	10.27 ± 1.04^{a}
IgM	42.23 ± 3.55^{ab}	39.19 ± 4.46^{a}	47.21 ± 3.62^{bc}	51.54 ± 4.74^{c}	46.45 ± 4.64^{b}	40.55 ± 2.96^{a}
DI						
LZ	168.41 ± 16.20^{b}	136.61 ± 12.49^{a}	213.57 ± 19.38^{c}	261.70 ± 14.50^{d}	198.88 ± 16.95^{c}	160.02 ± 14.19^{b}
AcP	236.44 ± 15.46^{b}	148.50 ± 11.09^{a}	297.32 ± 24.62^{c}	334.35 ± 12.11^{d}	368.72 ± 24.97^{e}	257.92 ± 18.94^{b}
C3	26.39 ± 1.96^{a}	24.42 ± 2.37^{a}	31.84 ± 2.93^{b}	37.09 ± 3.37^{c}	31.96 ± 2.96^{b}	27.23 ± 1.96^{a}
C4	11.50 ± 1.71^{b}	10.13 ± 0.92^{a}	13.70 ± 1.26^{c}	14.19 ± 0.85^{c}	12.93 ± 0.52^{c}	11.60 ± 1.02^{b}
IgM	40.48 ± 8.53^{bc}	34.59 ± 2.98^{a}	42.49 ± 2.77^{bc}	50.68 ± 2.57^{d}	44.88 ± 2.06^{c}	38.42 ± 3.63^{ab}

[a] Values are means ± SD (n = 6), and different superscripts in the same row are significantly different ($P < 0.05$). LZ, lysozyme (U/mg protein); AcP, acid phosphatase (U/mg protein); C3, complement 3 (mg/g protein); C4, complement 4 (mg/g protein); IgM, immunoglobulin M (mg/g protein).

upregulated. Interestingly, the IL-12p40 mRNA level was not influenced by dietary BA level ($P > 0.05$). The mRNA levels of IL-1β, IL-6 and IL-17D were lower in fish fed the LPHL diet than those of fish fed the NPNL diet with dietary BA levels up to 80 mg/kg diet ($P < 0.05$). In the DI, compared with the NPNL diet, fish fed the LPHL diet had higher mRNA levels of pro-inflammatory cytokines TNF-α, IL-6, IL-8 and IL-15 ($P < 0.05$). The mRNA levels of IL-1β, IFN-γ2, IL-12p35, IL-12p40 and IL-17D had no notable difference between the 2 groups ($P > 0.05$). The mRNA levels of IL-1β, TNF-α, IFN-γ2, IL-6, IL-8, IL-12p40 and IL-17D were all downregulated with dietary BA levels up to 160 mg/kg, and then upregulated. The mRNA levels of IL-12p35 and IL-15 were all downregulated with dietary BA levels up to 240 mg/kg, and then upregulated. With dietary BA levels up to 80 mg/kg diet, the mRNA level of IL-8 and IL-15 were comparable to those of the fish fed the NPNL diet, the mRNA level of TNF-α and IL-6 were significantly lower than those of fish fed the NPNL diet ($P < 0.05$).

As shown in Fig. 8. Compared with the NPNL diet, fish fed the LPHL diet had lower mRNA levels of anti-inflammatory cytokines IL-10 in the PI, and transforming growth factor β1 (TGF-β1) in the MI ($P < 0.05$). The mRNA levels of IL-10 (rather than PI), IL-11, IL-4/13A, IL-4/13B, TGF-β1 (rather than MI) and TGF-β2 in all three intestinal segments had no notable difference between the 2 groups ($P > 0.05$). The mRNA levels of IL-10, IL-11, TGF-β1 and TGF-β2 in all intestinal segments were all upregulated with dietary BA levels up to 160 mg/kg, and then downregulated. The mRNA level of IL-4/13A in all three intestinal segments were all upregulated with dietary BA levels up to 240 mg/kg, and then downregulated. However, no significant changes were observed for IL-4/13B mRNA levels ($P > 0.05$) in the PI, MI and DI of on-growing grass carp fed graded BA diets. With dietary BA levels up to 80 mg/kg diet, the IL-10 mRNA level in the PI and TGF-β1 in the MI were significantly higher than those of fish fed the NPNL diet ($P < 0.05$).

As shown in Fig. 9. Compared with the NPNL diet, fish fed the LPHL diet had higher mRNA levels of NF-κB p65, c-Rel, IκB kinases β (IKKβ), IKKγ, eIF4E-binding proteins 1 (4E-BP1) and 4E-BP2 in all intestinal segments and lower mRNA levels of inhibitor protein κBα (IκBα) and S6 kinases 1 (S6K1) in the PI and lower TOR in the PI and MI ($P < 0.05$). The mRNA levels of NF-κB p52 and IKKα in the three segments, IκBα and S6K1 in the MI and DI, and TOR in the DI had no notable difference between the 2 groups ($P > 0.05$). The mRNA levels of NF-κB p65, c-Rel, IKKβ, IKKγ and 4E-BP1 in all intestinal segments were all downregulated with dietary BA levels up to 160 mg/kg, and then upregulated. The mRNA levels of 4E-BP2 in all three intestinal segments were all downregulated with dietary BA levels up to 240 mg/kg, and then upregulated. Whereas, the mRNA levels of IκBα, TOR and S6K1 in all intestinal segments were upregulated with dietary BA levels up to 160 mg/kg, and then downregulated. Interestingly, NF-κB p52 and IKKα mRNA levels in the three segments were not influenced by dietary BA levels ($P > 0.05$). With dietary BA levels up to 80 mg/kg diet, the mRNA levels of IκBα and S6K1 in the PI, and TOR in the PI and MI, NF-κB p65, c-Rel (rather than MI), IKKβ, IKKγ, 4E-BP1 and 4E-BP2 in all three segments were comparable to those of the fish fed the NPNL diet, the mRNA level of c-Rel in the MI was significantly lower than those of fish fed the NPNL diet ($P < 0.05$).

3.7. Protein levels of NF-κB p65, p-TOR Ser2448 and T-TOR in the intestine of on-growing grass carp

As shown in Fig. 10 and Fig. 11, compared with the NPNL diet, on-growing grass carp fed the LPHL diet had higher protein levels of NF-κB p65 in all three intestinal segments ($P < 0.05$) and lower protein levels of p-TOR Ser2448/TOR in the PI and MI ($P < 0.05$). However, p-TOR Ser2448/TOR protein level in the DI had not notable difference in between the 2 groups. The protein levels of NF-κB p65 in the PI, MI and DI all decreased with increasing BA levels up to 160 mg/kg diet, above which the levels of these proteins increased. The protein levels of p-TOR Ser2448/TOR increased with increasing BA levels up to 160 mg/kg diet and then decreased in the three segments of on-growing grass carp. With dietary BA levels up to 80 mg/kg diet, the protein levels of NF-κB p65 in all three intestinal segments and p-TOR Ser2448/TOR in the MI were not significantly different from those in fish fed the NPNL diet, although the protein levels of p-TOR Ser2448/TOR in the PI were significantly higher than in those fed the NPNL diet ($P < 0.05$).

4. Discussion

4.1. The LPHL diet decreased the growth performance and intestinal immune function of fish

The present study shows that the LPHL diet decreased the growth

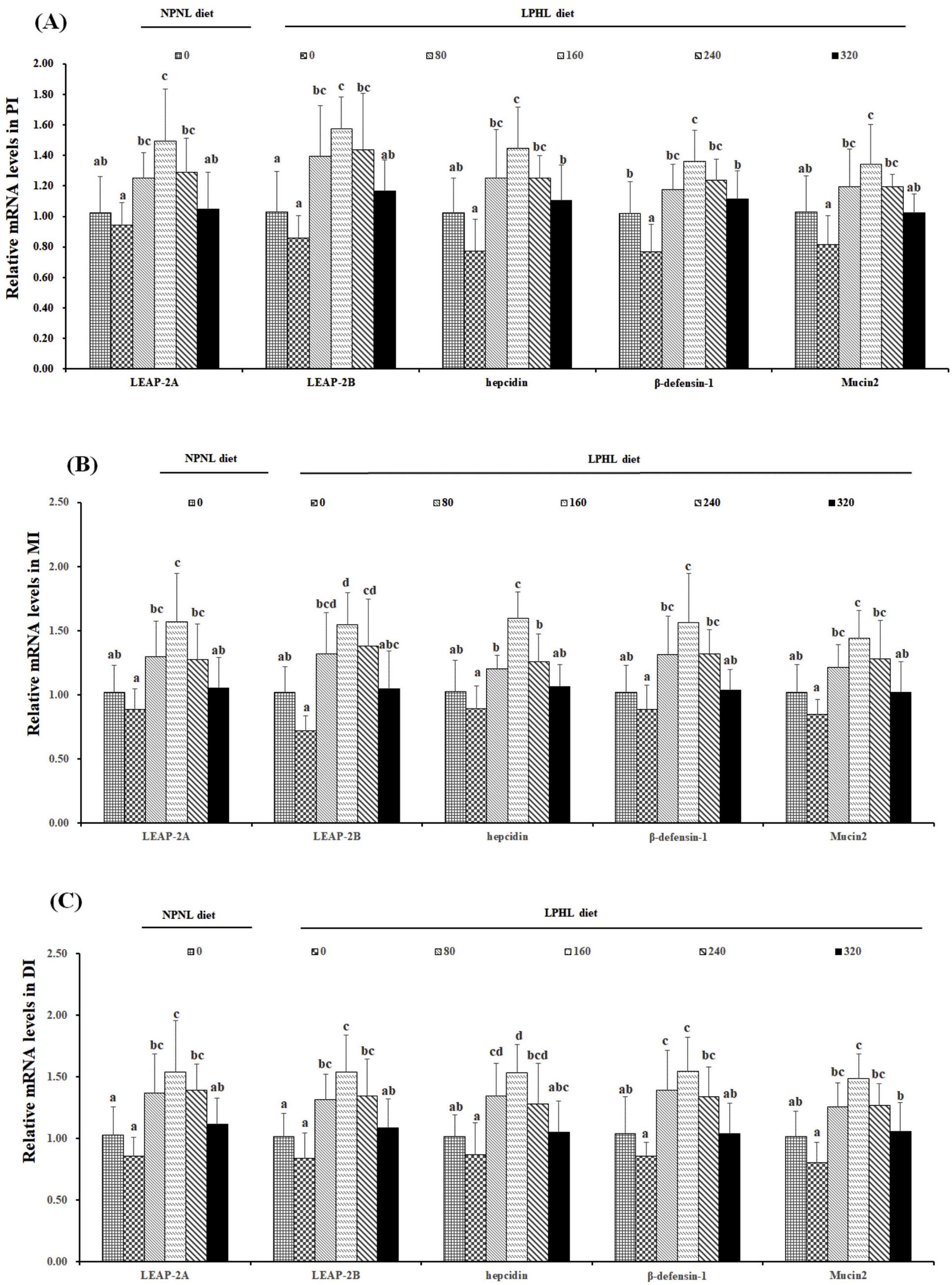

Fig. 6. Effects of dietary BA (mg/kg diet) supplementation on relative mRNA levels of LEAP-2A, LEAP-2B, hepcidin, β-defensin-1 and Mucin2 in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$), n = 6.

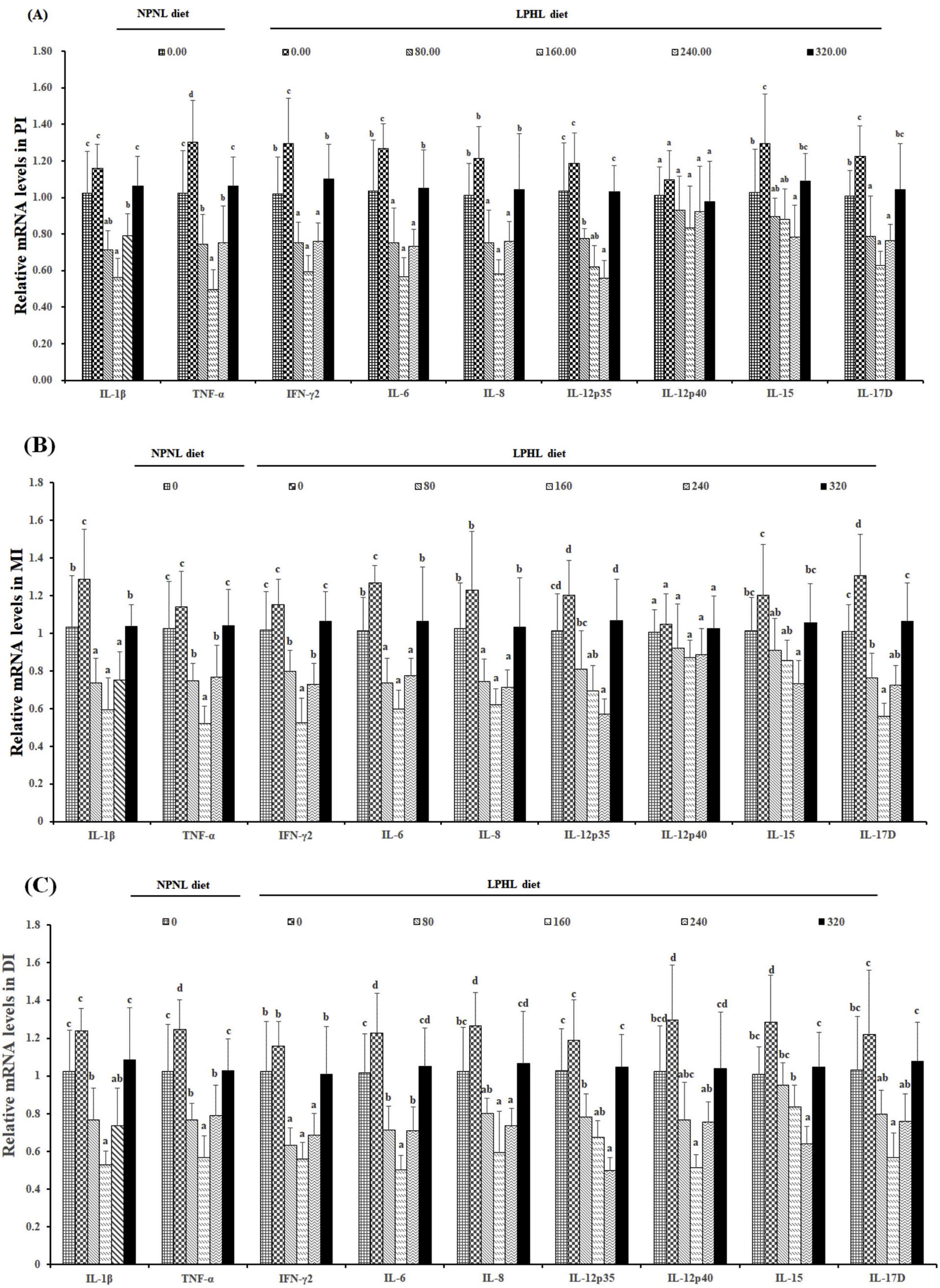

Fig. 7. Effects of dietary BA supplementation (mg/kg diet) in the LPHL diet on relative mRNA levels of pro-inflammatory cytokines in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$) n = 6.

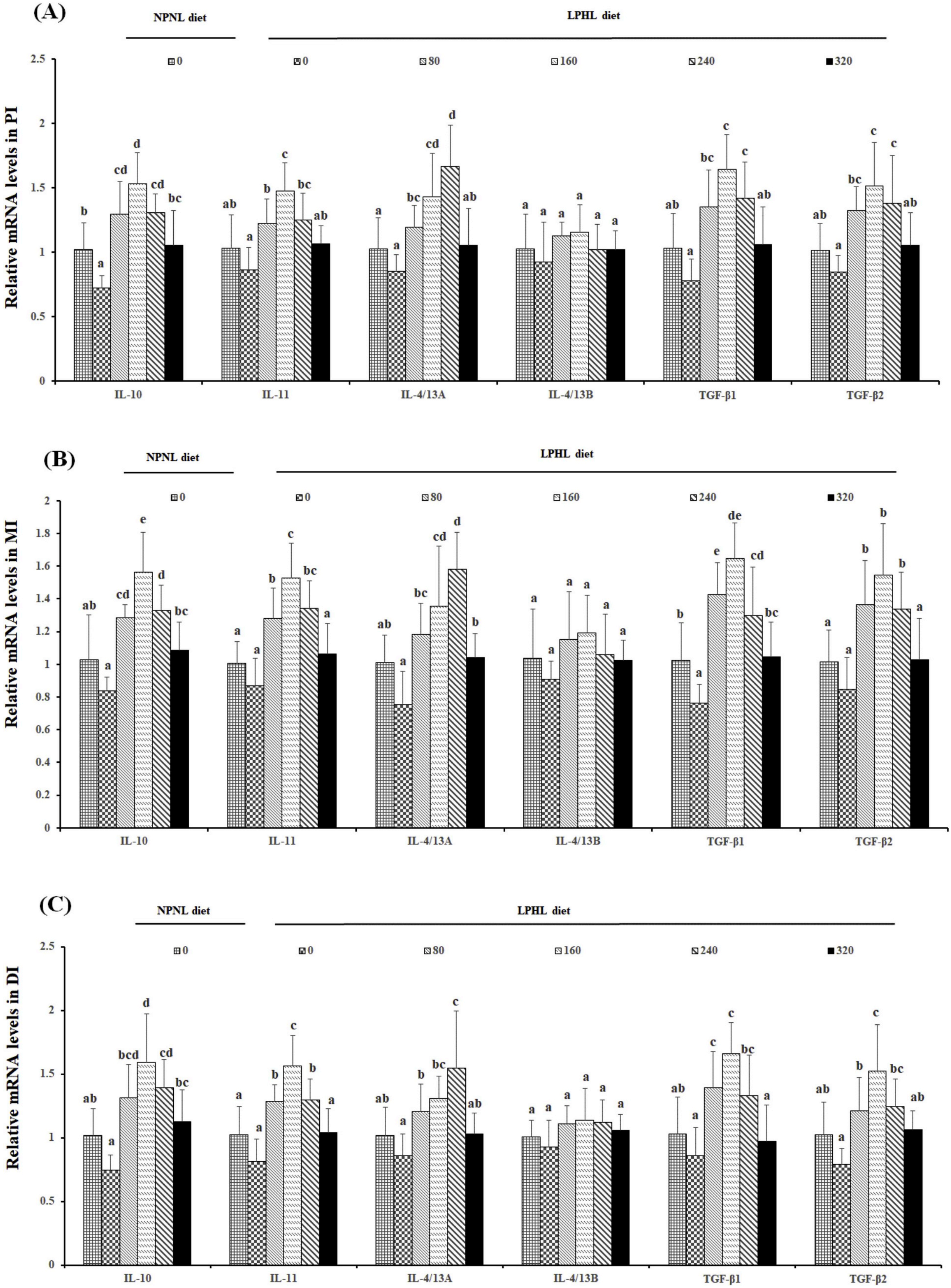

Fig. 8. Effects of dietary BA supplementation (mg/kg diet) in the LPHL diet on relative mRNA levels of anti-inflammatory cytokines in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). n = 6.

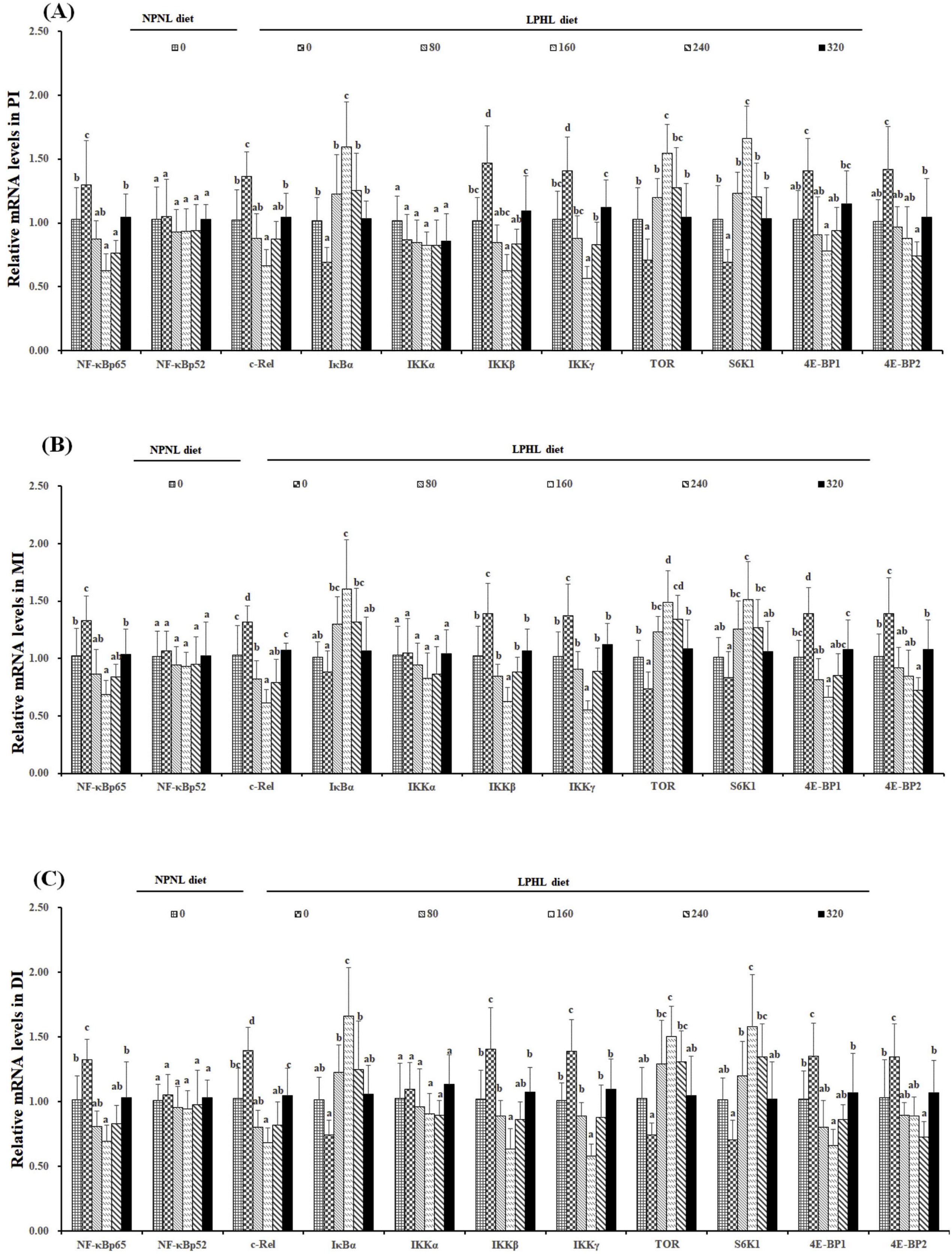

Fig. 9. Effects of dietary BA (mg/kg diet) supplementation in the LPHL diet on relative mRNA levels of signalling molecules in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). n = 6.

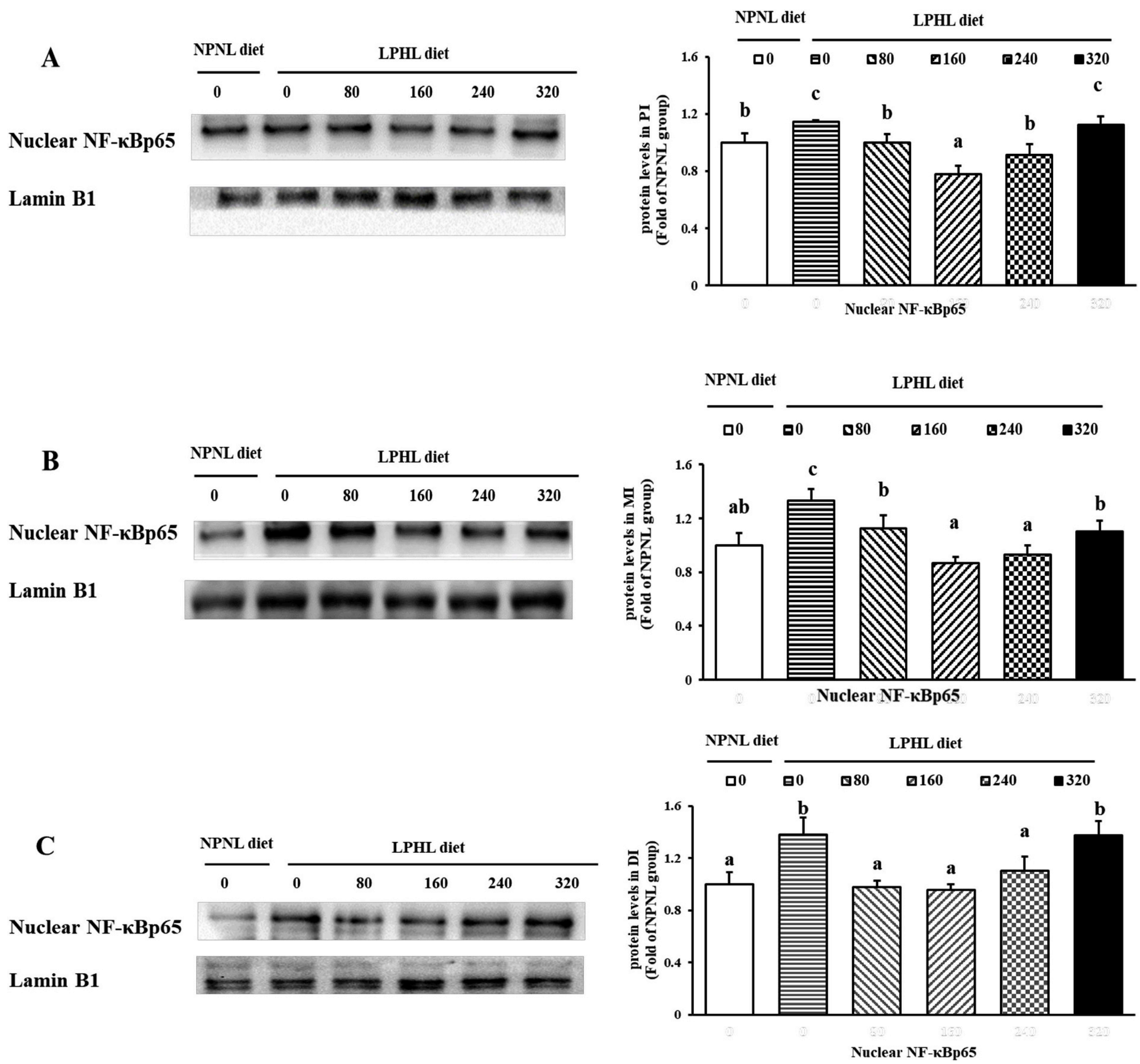

Fig. 10. Effects of dietary BA (mg/kg diet) supplementation in the LPHL diet on Western blot analysis of nuclear NF-κB p65 in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means of three replicates in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$), n = 3.

performance and intestinal growth of on-growing grass carp. Compared with the NPNL diet, the PWG, FI, IW and IL were significantly lower by approximately 14.52, 11.17, 12.98 and 21.36%, respectively. In fish, growth performance is closely associated with intestinal health [45], which is highly correlated to the balance of intestinal microflora [58] and intestinal immune function [59]. In the present study, compared with the NPNL diet, the LPHL diet disrupted the microbial balance (increased the number of beneficial bacteria *Lactobacillus* and *Bifidobacterium* and decreased the number of harmful bacteria *Aeromonas* and *E. coli*), increased the enteritis morbidity and decreased the intestinal immune function (e.g., decreased the IgM content and β-defensin-1 mRNA level in the PI) of on-growing grass carp. These adverse effects may be due to incomplete fat digestion. In the present study, the LPHL diet resulted in fatty liver disease, supporting this hypothesis. However, a study reported that supplementing BA in a high lipid diet could improve growth by promoting lipid digestion of juvenile grass carp [7]. Thus, we next examined the impact of exogenous BA supplementation in an LPHL diet on growth performance, intestinal growth and function, immune response, and related signalling pathway of fish.

4.2. Supplementation of BA in the LPHL diet improved fish growth performance and enteritis resistance capability

The present study clearly found that optimal BA supplementations in the LPHL diet enhanced the growth performance (FE, FI and PWG), improved the intestinal growth (IL, ILI and IW), relieved the fatty liver disease and enteritis symptoms (such as red and swollen intestines, blood capillary hyperaemia and lamina propria oedema) and decreased the enteritis morbidity of grass carp. For BA levels up to 80 mg/kg diet, the growth performance, intestinal growth and enteritis resistance capability were all superior to those of fish fed the NPNL diet. These data suggested that BA supplementations in the LPHL diet improved the resistance to enteritis in fish. In addition, enteritis resistance is associated with immune function, which is partially dependent on the innate and adaptive responses in the intestines of fish [60,61]. Therefore, we investigated the effect of dietary BA on the intestinal innate and adaptive responses of fish.

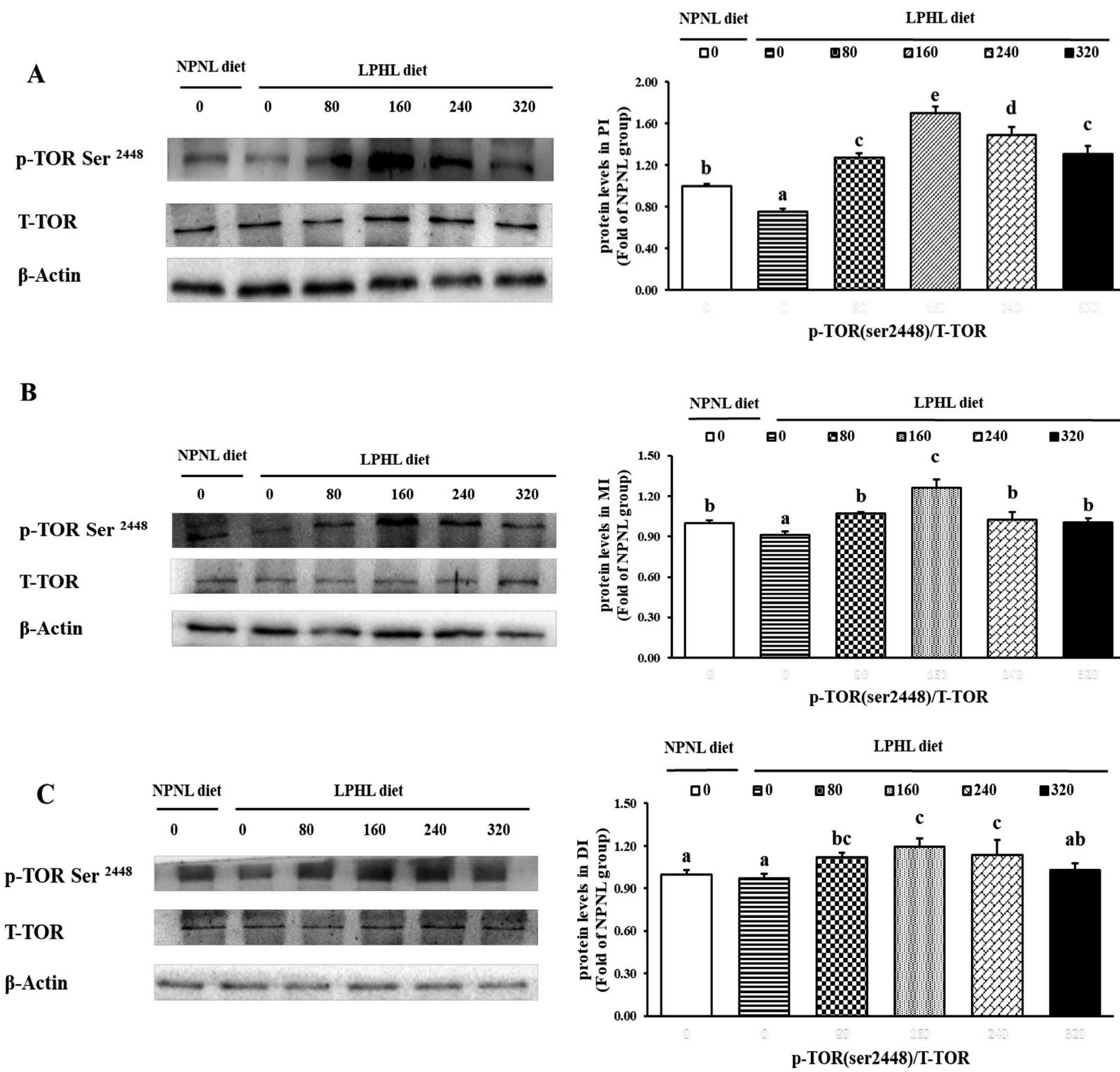

Fig. 11. Effects of dietary BA (mg/kg diet) supplementation in the LPHL diet on Western blot analysis of TOR protein phosphorylation (p-TOR Ser2448) in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means of three replicates in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$), n = 3.

4.3. Supplementation of BA in the LPHL diet increased fish intestinal immune function by improving production of innate and adaptive immune components

Immune function is related to innate and adaptive immune components, such as LZ, AcP, antimicrobial peptides and immunoglobulins in fish [62–64]. The present study clearly found that BA supplementations in an LPHL diet enhanced the immune function of intestine by promoting activities of AcP and LZ, the contents of IgM, C3 and C4, and the transcription of an antibacterial peptide (β-defensin-1, LEAP-2A, LEAP-2B, hepcidin and Mucin2). With dietary BA levels up to 160 mg/kg, the activities and contents of antibacterial compounds as well as the mRNA levels of antibacterial genes were higher than those of fish fed the NPNL diet. These results suggest that dietary BA supplementation strengthens the intestinal immune function partially associated with increasing innate and adaptive immune components of fish. Interestingly, our data found that the relative expression of immune-related genes was approximately 1–2-fold after infection with a nonlethal dosage of *A. hydrophila* in grass carp. Similar results have been also found in our laboratory's previous study on grass carp [28,65]. The fold change of immune-related gene relative expression may be related to the dose of *A. hydrophila* used to infect the fish. In Indian major carp (*L. rohita* Ham.), fish infected with 10^{10} CFU/ml *A. hydrophila* have higher immune responses than those infected with 10^{7} CFU/ml *A. hydrophila* [66]. In addition, immune responses is closely associated with the inflammatory response in the intestines of fish [67]. Hence, we next examined the effect of BA on the intestinal inflammatory response of fish.

4.4. BA supplementation in the LPHL diet attenuated the inflammatory response associated with NF-κB and TOR signalling pathways in the intestine of fish

It has been reported that inflammation can be activated by pro-inflammatory cytokines [68] and inhibited by anti-inflammatory cytokines [69], which could be regulated by the NF-κB family of transcription factors (such as NF-κB p65, p52 and c-Rel) and TOR/(S6K1, 4E-BP) signalling pathway in fish [70,71]. Several studies have reported that nuclear translocation of NF-κB is activated by IKKα, β, γ [72] and inhibited by IκBα [73]. The current study found that BA supplementation in an LPHL diet attenuated inflammation by downregulating IL-12p40 in the DI, IL-1β, TNF-α, IFN-γ2, IL-6, IL-8, IL-12p35, IL-15, IL-17D and NF-κB p65, c-Rel, IKKβ, γ and 4E-BP gene expression and NF-κB p65 protein levels in all three intestinal segments and by upregulating anti-inflammatory cytokine IL-10, IL-11, IL-4/13A, TGF-β1, TGF-β2, TOR, S6K1 and IκBα gene expression and p-TOR Ser2448/TOR protein levels in all three intestinal segments. Correlation analysis (Supplemental Table S2) showed that the mRNA levels of the pro-inflammatory cytokines (except IL-12p40 in the PI and MI) were positively correlated with NF-κB p65 protein levels and c-Rel mRNA levels. IκBα mRNA levels were negatively correlated with NF-κB p65, c-Rel, IKKβ and γ mRNA levels. Meanwhile, the mRNA levels of the anti-inflammatory cytokines (except IL-4/13B) were positively correlated with p-TOR Ser2448/TOR protein levels and S6K1 mRNA levels and were negatively correlated with 4E-BP mRNA levels. The above results suggest that BA supplementation in the LPHL diet attenuated the intestinal inflammatory response and may be partly affect the [(IKKβ and γ)/IκBα/(NF-κB p65 and c-Rel)] and [TOR/(S6K1, 4E-BP)] signalling pathways in the intestines of fish.

However, we found four interesting phenomena in cytokines and related signalling molecules. First, BA supplementation in the LPHL diet downregulated IL-12p40 mRNA levels in the DI (rather than the PI or MI) of on-growing grass carp, which might be partly associated with the farnesoid X receptor (FXR) and the glucocorticoid receptor. A previous study revealed that BA could activate the FXR in rat small intestine (IEC-6) cells [74], which could increase the glucocorticoid receptor mRNA level in the HepG2 cell line [75]. In peripheral blood mononuclear cells, the glucocorticoid receptor suppresses IL-12p40 production [76]. Nevertheless, a study in tilapia (*Oreochromis mossambicus*) found that glucocorticoid receptor mRNA in the DI is higher than that in the PI and MI [77]. Hence, we hypothesize that BA supplementations downregulated IL-12p40 mRNA levels in the DI (rather than the PI and MI) partially due to higher mRNA levels of the glucocorticoid receptor in the DI compared to the PI and MI of fish. However, this hypothesis needs further investigation. Second, BA supplementation in the LPHL diet upregulated the mRNA levels of IL-4/13A (rather than IL-4/13B) in all three intestinal segments of on-growing grass carp, which might be partly related to TOR and GATA3. Our data found that optimal BA levels promote TOR activation in all three intestinal segments of on-growing grass carp. Cook and Miller revealed that activation of mTOR enhances GATA-3 protein expression in mice $CD4^+$ cells [78]. It was reported that GATA3 could regulate the gene transcription of IL-4/13A through binding with a TATA box [79], which was located in IL-4/13A (rather than IL-4/13B) of pufferfish (*Tetraodon nigroviridis*) [80]. Hence, we hypothesis that BA upregulated TOR mRNA levels leading to the upregulation of GATA-3, which could bind with the TATA box in the IL-4/13A gene (rather than IL-4/13B) and might ultimately result in the upregulation of IL-4/13A (rather than IL-4/13B) mRNA levels in the intestine of fish. However, this underlying reasoning requires further study. Third, BA supplementation in the LPHL diet had no impact on NF-κB p52 mRNA levels in all three intestinal segments of on-growing grass carp, which might be partly related to the unaffected IKKα mRNA levels. It has been confirmed that IKKα is crucial for the activation of NF-κB p52 in mammalian cells [81]. The present study showed that BA had no impact on the mRNA levels of IKKα in all three intestinal segments of on-growing grass carp, which supported our hypothesis. Finally, downregulating of IKKβ and IKKγ (not IKKα) mRNA levels due to BA supplementation in the LPHL diet might be partially related to TNF-α and protein kinase Czeta (PKC-ζ). In the present study, BA supplementations downregulated TNF-α mRNA levels in the intestines of on-growing grass carp. A study in rats reported that a reduction in TNF-α expression could decrease PKC-ζ activity [82]. The decrease of PKC-ζ activity could downregulate IKKβ and IKKγ (not IKKα) expression in rat Kupffer cells [83]. Hence, we hypothesize that BA supplementations downregulated the TNF-α mRNA level leading to the decrease in PKC-ζ activity, resulting in the downregulation of IKKβ and IKKγ (not IKKα) mRNA levels in the intestine of fish. However, this hypothesis needs further investigation.

4.5. Physiological mechanism of action of BA

Based on the above, BA can improve fish growth performance and enteritis resistance capability, increase fish intestinal immune function, and attenuate fish intestine inflammatory response. The physiological mechanism of action of BA may be partially explained two ways. First, it might be related to lipid metabolism. As we all know, BA could enhance the digestion of lipids by acting as emulsifying agents that render fats accessible to pancreatic lipases [84] and prevent fatty liver [42]. Data from studies conducted with *Anguilla anguilla* L. [85], *Paralichthys olivaceus* [86], *Schizothorax prenanti* [87] and grass carp [7] indicate that BA can enhance lipase activity and gene expression, which could improve lipid digestion, as has been reported in higher vertebrates [88]. In the present study, supplementation of 160 mg/kg BA in the LPHL diet prevented fatty liver disease, supporting our hypothesis. Second, it might be related to modifying the immune response via activating TGR5. In addition to the role of BA in dietary lipid metabolism, BA also function as signalling molecules capable of activating specific receptors (e.g., TGR5) [8]. BA could inhibit LPS-induced production of several pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, by activating TGR5 in alveolar macrophages [89]. In the present study, optimal BA supplementation levels decreased the mRNA levels of TNF-α, IL-1β, IL-6, and IL-8 in the intestine of grass carp, supporting this hypothesis. Hence, the physiological mechanism of action of BA might be attributed to the facilitated lipid metabolism and modified the immune response via activation TGR5.

4.6. Negative effects of high levels of BA

However, the present study found that, compared with optimal exogenous BA supplementation, high levels of exogenous BA supplementation (320 mg/kg diet) decreased growth performance and intestinal immunity in on-growing grass carp. The possible reasons for the differences are outlined below. First, excessive exogenous BA might impair the enterohepatic circulation of BA. In rats, the addition of lithocholic acid (LCA, a secondary BA) to the diet leads to gallstone formation because supplementation with LCA increases the demand of taurine for conjugate production and brings about taurine depletion [90]. As a result, LCA and its metabolite were conjugated with glycine, and then precipitated in bile as insoluble calcium salts [91]. In the present study, supplementation of 320 mg/kg BA in the LPHL diet resulted in a changed gall bladder morphology. In a future study, analysis of the gall bladder compositions might verify this hypothesis. Second, the negative effects of high levels of BA may be related to cholesterol accumulation. In tilapia, high levels of BA caused accumulation of serum cholesterol [42]. High levels of cholesterol impaired immunity and aggravated the inflammation response in grass carp [25]. Third, it could be attributed to the cytotoxicity to hepatocytes of BA [92]. In mice, it was reported that dietary LCA supplementation resulted in intrahepatic cholestasis and bile infarcts [93], and the accumulation of BA was a major mediator of cholestatic liver injury in humans [94]. Jiang et al. [42] found that an excess of exogenous BA in tilapia resulted in serious nuclear migration and vacuolization and enhanced ALT and AST activities, which suggested that liver cells were partly damaged in the hepatocytes. Fourth, the adverse effects may be related to the high levels of free fatty acids (FFAs). FFAs are the primary lipolysis products by lipids [95]. In rats, ursodeoxycholic acid (a secondary BA) could increase saturated and unsaturated acid levels [96,97]. In human trophoblasts, saturated fatty acids could induce inflammatory effects by activating the TLR4 and NF-κB signalling pathway [98]. Excess n-3 PUFAs in diets inhibited the expression levels of LZ mRNA and LZ activities in sea cucumbers *Apostichopus japonicus* (Selenka) [99]. A study on grass carp found that an impaired intestinal immune system caused

Table 4
The optimal dietary BA levels in the LPHL diet based on PWG, FI, the enteritis morbidity and activities of LZ in MI and AcP in the DI of on-growing grass carp (*Ctenopharyngodon idella*).

Indices	Regression equation	R^2	*P*	Optimal BA levels
PWG	$y = -0.0026x^2 + 0.8787x + 112.28$	0.9527	< 0.05	168.98 mg/kg diet
FI	$y = -0.0047x^2 + 1.6002x + 414.97$	0.9611	< 0.05	170.23 mg/kg diet
the enteritis morbidity	y = 3E-06x^2 - 0.001x + 0.1916	0.9084	= 0.092	166.67 mg/kg diet
LZ in the MI	$y = -0.0025x^2 + 0.8825x + 114.2$	0.9606	< 0.05	176.50 mg/kg diet
AcP in the DI	$y = -0.0058x^2 + 2.2268x + 148.75$	0.9648	< 0.05	191.97 mg/kg diet

decreased growth performance [100]. As far as the above observations are concerned, we conclude that the decreased growth performance of fish that ingested high levels of exogenous BA may be partly related to its adverse effects on enterohepatic circulation of BA, accumulation of cholesterol, cytotoxicity to hepatocytes and high levels of FFAs. However, this observation warrants future investigation.

4.7. Optimal BA supplementation in the LPHL diet

The present study showed that, compared with the NPNL diet, the LPHL diet decreased the growth performance and intestinal immunity of on-growing grass carp, whereas exogenous BA supplementation in the LPHL diet could reverse this negative effect. Hence, in the LPHL diet, calculating the level of BA supplementation equal to that in the NPNL diet has important production significance. In this study, based on quadratic regression analysis, with dietary BA levels up to 26.44, 37.43, 23.22, 33.60 and 44.55 mg/kg in the LPHL diet, the PWG, FI, enteritis resistance capacity and activities of LZ in the MI and AcP in the DI reached equivalent levels to those of the NPNL diet; therefore, in the LPHL diet, the comparable level of BA supplementation relative to the NPNL diet of on-growing grass carp was estimated to be 23.22–44.55 mg/kg. Simultaneously, we investigated the optimal levels of BA supplementation for on-growing grass carp based on the previous indices in the LPHL diet. With dietary BA levels up to 168.98, 170.23, 166.67, 176.50 and 191.97 mg/kg (Table 4), the aforementioned indices reached optimal levels and even surpassed the levels reached in the NPNL diet, indicating that BA supplementation in the LPHL diet could enhance the effect of protein-sparing by dietary lipids and improve the growth performance and intestinal immunity in on-growing grass carp. Interestingly, we found that the optimal BA level were higher (166.67–191.97 mg/kg diet) than the levels of BA supplementation comparable to the NPNL diet (23.22–44.55 mg/kg diet), demonstrating that BA may have additional benefits, such as improving intestinal health and enhancing the resistance to enteritis by promoting the balance of intestinal microflora in fish. In addition, our results showed that the optimal BA level for enhancing immune function was higher than that for promoting growth performance in fish. This difference might be related with the BA receptor FXR. It has been reported that FXR alleviates inflammation via reducing production of pro-inflammatory cytokines in mice [101]. Hence, we suggest that more BA need to be supplied for the development of the immunity of the intestine by improving immune function.

5. Conclusions

In summary, the present study indicated that the LPHL diet decreased growth performance and enteritis resistance capability and partly impaired the immune function of the intestine in grass carp, whereas BA supplementation (80–320 mg/kg) in the LPHL diet could completely reverse this adverse effect through a mechanism that could partly be attributed to the following: (i) the increase in innate and adaptive immune components, including LZ and AcP activities, C3, C4 and IgM contents and the upregulation of hepcidin, LEAP-2A, LEAP-2B, β-defensin-1 and Mucin2 mRNA levels; and (ii) the downregulation of pro-inflammatory cytokines, including IL-1β, TNF-α, IFN-γ2, IL-6, IL-8, IL-12p35, IL-12p40 (only in the DI), IL-15 and IL-17D, which are partially involved in the downregulation of IKKβ and IKKγ (rather than IKKα), NF-κB p65 and c-Rel (rather than NF-κB p52) mRNA levels and the upregulation of IκBα and anti-inflammatory cytokine IL-10, IL-11, IL-4/13A, TGF-β1, TGF-β2 and S6K1 mRNA levels partially associated with the TOR signalling pathway [TOR/S6K1, 4E-BP1 and 4E-BP2]. Finally, based on PWG, FI, protection against enteritis morbidity and the immune index (LZ activity in the MI and AcP activity in the DI), the optimal BA supplementation in the LPHL diet for on-growing grass carp were estimated to be 168.98, 170.23, 166.67, 176.50 and 191.97 mg/kg diet, respectively.

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Appendix A. Supplementary data

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