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Phytic acid disrupted intestinal immune status and suppressed growth performance in on-growing grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Phytic acid (PA) is one of the most common anti-nutritional factors in plant-derived protein feeds, and it poses considerable threats to aquaculture production. However, little is known about the effects of PA on fish intestinal health. This study aimed to investigate the impacts of PA on intestinal immune function in on-growing grass carp. To achieve this goal, a growth trial was conducted for 60 days by feeding 540 fish (120.56 ± 0.51 g) with six semi-purified diets containing graded levels of PA (0, 0.8, 1.6, 2.4, 3.2 and 4.0%). Then fish were challenged with *Aeromonas hydrophila* for 6 days. The results indicated that, compared with the control group (0% PA), PA did the following: (1) suppressed fish growth performance (percentage weight gain and feed efficiency) and reduced their ability to resist enteritis; (2) decreased fish intestinal antimicrobial ability by reducing intestinal lysozyme (LZ) activities, the contents of complement 3 (C3), C4 and immunoglobulin M (IgM), and down-regulating the mRNA levels of hepcidin, liver-expressed antimicrobial peptide 2A (LEAP-2A), LEAP-2B, and β-defensin-1; and (3) aggravated fish intestinal inflammation responses by upregulating the mRNA levels of pro-inflammatory cytokines including tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) (except in the DI), interferon γ2 (IFN-γ2), IL-8, IL-12p40, IL-15 (except in the DI) and IL-17D, which is partly related to the nuclear factor kappa B (NF-κB) signalling pathway, whereas downregulating the mRNA levels of anti-inflammatory cytokines including transforming growth factor β1 (TGF-β1), IL-4/13A, IL-4/13B, IL-10 and IL-11, which is partially associated with the target of rapamycin (TOR) signalling pathway. The possible reasons for some distinctive gene expression patterns in fish three intestinal segments were discussed. Finally, based on the percent weight gain, enteritis morbidity, IgM content and LZ activity in the PI, the maximum tolerance levels of PA for on-growing grass carp were estimated to be 2.17, 1.68, 1.47 and 1.18% of the diet, respectively.

1. Introduction

Fish intestines constitute the first line of defence, protecting organisms from various pathogens in the environment [1,2]. It has been reported that the disturbance of intestinal immune function could lead to enteritis in fish [3], which has been considered one of the major threats to the healthy growth of fish under intensive culture conditions [4,5]. Plant protein sources such as soybean meal, rapeseed meal and cottonseed meal have been recognised to be appropriate in aquaculture feeds [6]. However, a high proportion of plant proteins used in diets caused enteritis in several fish species [7–9]. The inflammatory effects might be partly associated with the presence of anti-nutritional factors (ANFs) in plant ingredients, as ANFs such as saponins, phytosterols, soybean β-conglycinin and gossypol in diets have been reported to induce enteritis in fish [10–13]. Phytic acid (PA) is known as one of the major ANFs in plant-derived proteins [14], occupying approximately 1.0–1.5, 5.0–7.5 and 2.4–4.4% of soybean meal, rapeseed meal and cottonseed meal, respectively [14–16]. Little is known about the effects of PA on fish intestinal health. Only two studies have reported that PA caused anomalies in the pyloric caecal structure of juvenile chinook

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salmon (*Oncorhynchus tshawytscha*) [17] and increased acid phosphatase (ACP) activity in the mid intestine of Atlantic salmon (*Salmo salar* L.) [18]. Therefore, a systematic investigation on the effects of PA on fish intestinal immune function is needed, and the potential molecular mechanisms should be illuminated.

Fish intestinal immune function is tightly associated with various immune components such as lysozyme (LZ), antimicrobial peptides and immunoglobulin M (IgM) [19,20]. However, except for the reported ACP, no evidence has been reported about the effects of PA on these immune components in fish intestines. In grass carp, PA reduced the serum concentration of Zn [21], while Zn deficiency could decrease fish intestinal LZ activity [22]. Meanwhile, Gu et al. [23] reported that PA inhibited c-fos in PC-3 cells. In rat hepatocytes, c-fos inhibition could downregulate hepcidin gene expression [24]. Additionally, in mice, PA enhanced natural killer (NK) cell activity [25], which could suppress the IgM production of B cells [26]. These results indicate that PA may affect the intestinal immune components of fish, which deserves investigation.

Except for immune components, the inflammatory response also plays a significant role in fish intestinal immune function [27]. However, immoderate inflammation disrupts the immune function and causes the organism to become more susceptible to pathogens [28]. Cytokines, categorised into pro-inflammatory cytokines [e.g., tumour necrosis factor α (TNF-α)] and anti-inflammatory cytokines [e.g., transforming growth factor β (TGF-β)], are the key mediators of inflammation [29] and are regulated by the nuclear factor kappa B (NF-κB) and target of rapamycin (TOR) signalling pathways in fish [30,31]. No studies have yet reported the relationship between PA and fish intestinal inflammation. In the ileum of piglets, dietary PA reduced Na^+-dependent glucose transporter 1 (SGLT1) gene expression [32], while the genetic knockout of SGLT1 in mice upregulated the hepatic mRNA levels of TNF-α [33]. In rats, PA diminished the serum concentration of thyroid hormone [34], and decreased thyroid hormone could lower the mRNA level of TGF-β in cardiomyocytes [35]. Further, PA reduced the serum alkaline phosphatase (AKP) activity in juvenile grass carp [21]. Decreased AKP activity led to the activation of the NF-κB signalling pathway in IEC-6 cells [36]. In addition to these results, PA was reported to block the phosphatidylinositol-3 kinase (PI–3K) activity in JB6 cells [37], while PI-3K inhibition could suppress the mTOR signalling pathway in TU212 cells [38]. Based on the observations above, we assume that PA may affect the fish intestinal inflammation that is partly associated with the regulation of the TOR and NF-κB signalling pathways, which is worthy of exploration.

Grass carp (*Ctenopharyngodon idella*) is an economically important farm fish in China [39]. The national production of grass carp exceeded 5.3 million tons in 2017, accounting for approximately 10% of the country's total aquaculture production [40]. Grass carp has also been introduced to more than 40 countries [41]. The global production of grass carp reached approximately 6.1 million tons or 12.1% of worldwide freshwater aquaculture production in 2016 [42]. However, intensively farmed grass carp are frequently infected by bacterial, viral, and parasitic pathogens, making them susceptible to various diseases, such as rotten gill disease, haemorrhagic disease, enteritis and septicaemia [5,43]. The bacterial enteritis caused by *Aeromonas hydrophila* has been recognised to be probably the most widespread disease in grass carp under intensive culture. This disease causes huge economic losses annually due to the suppression of fish growth and high mortality rates [44]. It is worth mentioning that *A. hydrophila* is an opportunistic pathogen, which means it does not cause problems in conditionally healthy fish [45]. Hence, maintaining grass carp intestinal immune function and resistance against this pathogen is of great importance to developing healthy grass carp culture. In the present study, we examined for the first time the effects of PA on grass carp intestinal immune function under the stress of *A. hydrophila*, which is related to immune components, cytokines and the related signalling pathways. The results provide partial theoretical evidence for the influence of PA on fish intestinal immune function and its possible molecular mechanisms. The diets of grass carp contain high proportions of plant feedstuffs [46], which could be abundant in PA [14]. Hence, the maximum tolerance levels of PA for grass carp based on the intestinal health and growth performance were estimated, which could provide a novel reference for commercial feed formulations.

Table 1
Composition and nutrients content of the basal diet.

Ingredients	%	Nutrients content	%
Fish meal	6.50	Crude protein[c]	28.78
Casein	22.70	Crude lipid[c]	4.95
Gelatin	6.00	n-3[d]	1.04
DL-Met (99%)	0.44	n-6[d]	0.96
L-Trp (99.2%)	0.07	Available phosphorus[e]	0.40
Fish oil	2.40		
Soybean oil	1.81		
α-starch	22.00		
Corn starch	22.56		
Cellulose	5.00		
$Ca(H_2PO_4)_2$	1.47		
Vitamin premix[a]	1.00		
Mineral premix[b]	2.00		
Ethoxyquin (30%)	0.05		
Choline chloride (50%)	1.00		
PA premix	5.00		

[a] Vitamin premix (g kg^{-1}): retinyl acetate (500,000 IU g^{-1}), 0.39; cholecalciferol (500,000 IU g^{-1}), 0.20; D, L-a-tocopherol acetate (50%), 23.23; menadione (22.9%), 0.83; thiamine nitrate (98%), 0.10; calcium-D-pantothenate (98%), 4.18; pyridoxine hydrochloride (98%), 0.45; cyanocobalamin (1%), 0.94; niacin (99%), 3.44; D-biotin (2%), 0.75; meso-inositol (98%), 28.23; folic acid (95%), 0.17; riboflavin (80%), 0.73; ascorhyl acetate (95%), 9.77. All ingredients were diluted with corn starch to 1 kg.

[b] Mineral premix (g kg^{-1}): $MnSO_4.H_2O$ (31.8% Mn), 2.6590; $MgSO_4.H_2O$ (15.0% Mg), 200.0000; $FeSO_4.H_2O$ (30.0% Fe), 12.2500; $ZnSO_4.H_2O$ (34.5% Zn), 8.2460; $CuSO_4.5H_2O$ (25.0% Cu), 0.9560; KI (76.9% I), 0.0668; Na_2SeO_3 (44.7% Se), 0.0168. All ingredients were diluted with corn starch to 1 kg.

[c] Crude protein and crude lipid contents were measured values.

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

[e] Available phosphorus were referenced to Wen et al. [104] and calculated according to NRC (2011).

2. Materials and methods

2.1. Experimental diets and procedures

The composition and nutrient level of a basal diet are presented in Table 1. Fish meal, casein, and gelatine were used as the protein sources. Fish oil and soybean oil were used as the lipid sources. Phytic acid was supplemented in the basal diet to obtain 0 (as the control group), 0.8, 1.6, 2.4, 3.2 and 4.0% of PA in the experimental diets, and the final amounts in the different diets were compensated for by using corn starch according to Rasid et al. [47]. The diets were produced as pellets and then stored at 4 °C in plastic bags until used as recommended by Chowdhury et al. [48].

2.2. Growth trial and sample collection

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University. The grass carp obtained from a local fishery (Sichuan, China) were acclimatised for 4 weeks in an experimental environment according to Xu et al. [49]. Then, 540 healthy fish (average weight 120.56 ± 0.51 g) were selected and randomly distributed into 18 experimental cages (1.4 L × 1.4 W × 1.4H m), with 30 fish in each cage. All cages were randomly assigned to 6 treatments giving each treatment three replicates. Fish were fed with corresponding diets to apparent satiation

four times per day. At the bottom of each cage, we furnished a round plate (diameter 100 cm) to collect the residual feed left by the fish according to Tang et al. [50]. Approximately 30 min after each feeding, the residual feed was collected, dried and weighed for the calculation of feed intake (FI). The growth trial lasted for 60 days. During the trial, the oxygen content in the water was over 6.0 mg L^{-1}, and the water temperature and pH were determined to be 25.6 ± 2.2 °C and 7.5 ± 0.3, respectively. The trial was conducted under natural light cycles.

At the beginning and end of the trial, fish were weighed for the calculation of growth performance parameters. After weighing, fifteen fish were randomly captured from each treatment and then sacrificed after anaesthesia with benzocaine as described by Geraylou et al. [51]. After gut opening, fish intestines were rapidly removed, and their contents (faecal matter and undigested feed) were carefully squeezed out according to Galagarza et al. [52]. The intestines were then segmented into proximal intestine (PI), mid intestine (MI) and distal intestine (DI). The length of each intestinal segment was measured. Intestinal histological samples from 3 fish in each group were stored in 4% paraformaldehyde solution for slices.

2.3. The establishment of A. hydrophila infection model

To establish an appropriate *A. hydrophila* infection model, both the challenge dose and the period are essential [5]. We first conducted a pre-experiment to determine an appropriate challenge dose as recommended by Song et al. [5]. The *A. hydrophila* used in the present study was obtained from the College of Veterinary Medicine, Sichuan Agricultural University. A total of 90 healthy fish (mean weight 433.8 ± 20.9 g) were selected and divided randomly into 6 treatments with 15 fish in each treatment. After fasting for 24 h, fish in each group were injected intraperitoneally with 1.0 ml of *A. hydrophila* kg^{-1} fish [53]. The concentrations of *A. hydrophila* were 0 (physiological saline), 1.5×10^6, 1.5×10^7, 1.5×10^8, 1.5×10^9 and 1.5×10^{10} colony-forming units (cfu)·ml^{-1}. The symptoms and the mortality of the fish after infection were recorded daily. The experimental conditions were the same as in the growth trial. Finally, the challenge dose was confirmed to be 1.5×10^8 cfu ml^{-1}, which could effectively induce inflammation without causing any death within 15 days (S Fig. 1).

Then, an appropriate challenge period was selected by conducting another pre-experiment. Another 90 healthy fish (mean weight 442.1 ± 19.6 g) were randomly divided into 6 groups and injected intraperitoneally with 1.0 ml of *A. hydrophila* at a dose of 1.5×10^8 cfu ml^{-1}·kg^{-1}. During the test, the symptoms and mortality of fish were monitored daily. Six groups of fish were punctually sampled at the experimental periods of 0 (right after the injection), 3, 6, 9, 12 and 15 days after injection. The intestines of the fish were quickly removed and segmented, and the severity of enteritis was then scored based on the degree of intestinal swelling and red coloration according to Song et al. [5] and David et al. [54]. The indices of the intestinal segments, which include LZ and ACP activities, C3, C4 and IgM contents, and the mRNA levels of IL-10, TGF-β1, TGF-β2, TNF-α and IL-1β, were analysed. The methods used in these analyses were the same as those from biochemical analysis and real-time polymerase chain reaction (PCR) analysis.

2.4. Challenge trial

Our data in the second pre-experiment displayed that at the 6th day after challenge, most of the immune components and all of the pro-inflammatory cytokines reached the highest levels (S Table 1 and Fig. 1), which indicates the most severe inflammatory responses within 15 days. Based on this result, we selected the challenge period to be 6 days. After a 60-day growth trial, 24 fish were randomly captured from each treatment to conduct a challenge trial by injecting with *A. hydrophila* intraperitoneally. Fish from the control group were captured and injected with physiological saline to serve as the control group of challenge trial. Each individual was injected with 1.0 ml of 1.5×10^8 cfu ml^{-1}·kg^{-1} *A. hydrophila* or physiological saline for 6 days. The dose and period of the challenge trail were both appropriate to induce inflammation effectively without causing any death. After the challenge trial, fish were sacrificed after anesthetisation with benzocaine. Fish intestines were promptly separated, squeezed and segmented and then scored for the severity of enteritis. After that, the samples of fish intestines were quickly frozen in liquid nitrogen and saved at −80 °C for biochemical analysis.

2.5. Biochemical analysis

The approximate compositions of the diets were analysed according to AOAC [55]. Briefly, the micro-Kjeldahl method and the Soxhlet method were used for crude protein and crude lipid determination, respectively. Intestinal samples were homogenised in 9 vol (w/v) of ice-cold physiological saline and then centrifuged at 6000 g for 20 min at 4 °C to obtain the supernatant for subsequent analysis. The LZ activity was determined using the method described by Deng et al. [56]. The ACP activity was analysed according to Molina et al. [57]. The levels of C3, C4 and IgM were assayed using corresponding kits (Elikan, Wenzhou, Zhejiang, China). The content analysis of C3, C4 and IgM measured the turbidity increase after the immunity response according to Sun et al. [58] and Xu et al. [49].

2.6. Histological examination

The intestinal segments preserved in 4% paraformaldehyde solution were clear with physiological saline and dehydrated through a series of concentrations of alcohols (from 70% to absolute). Tissues were embedded in paraffin wax, cut at 4 μm, and then stained with haematoxylin and eosin (H & E) as described by Adil et al. [59]. The histological sections were examined by using a light microscope (Nikon Eclipse TS100, Nikon Corporation, Tokyo, Japan). To estimate the proportion of each symptom in the three fish intestinal segments, a scoring system recommended by Liu et al. [60] was used in the present study. Briefly, 10 images were randomly selected from each fish in each group (0, 2.4 and 4.0% diet). Then, the symptoms were scored as follows: 0 = not observed; 1 = low frequency (1–3 out of 10 images); 2 = moderate frequency (4–6 out of 10 images) and 3 = high frequency (≥7 out of 10 images).

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

The total RNA was extracted with an RNAiso Plus kit (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's instructions, followed by a DNAse I treatment. The integrity of RNA was assessed by agarose gel (1%) electrophoresis, and the RNA quantity was analysed by using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) according to Smith et al. [61]. Subsequently, cDNAs were synthesised using a PrimeScript™RT reagent kit (TaKaRa Biotechnology Co. Ltd, Dalian, Liaoning, China). Real-time PCR analysis was conducted with a CFX96™ Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.). The specific primers for PCR were designed based on the sequences published and cloned in our laboratory of grass carp (Table 2). Based on the evaluation of internal control genes in our preliminary experiment, β-actin was selected to be the reference gene to normalise cDNA loading. The efficiency of amplifying the target and housekeeping genes was calculated based on the specific gene standard curves, which were generated from 10-fold serial dilutions. After verifying that the efficiency of the amplifying primers reached approximately 100%, the $2^{-\Delta\Delta CT}$ method was used to analyse the final expression results as described by Livak and Schmittgen [62].

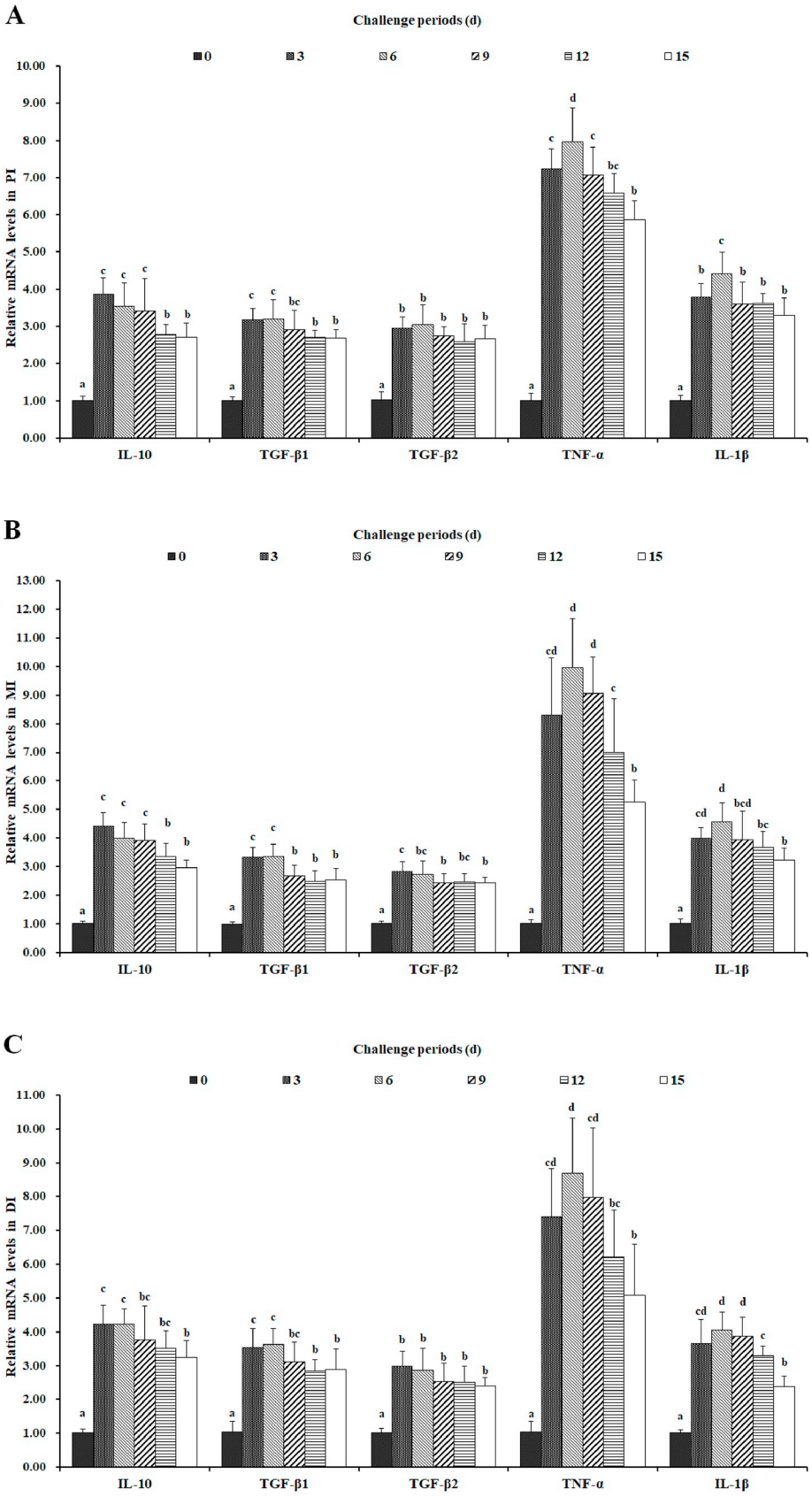

Fig. 1. Relative mRNA levels of pro-inflammatory and anti-inflammatory cytokines in the PI (A), MI (B) and DI (C) of grass carp after challenge with 1.5×10^8 cfu/ml *Aeromonas hydrophila* for different periods. Data represent means (n = 6 individuals in each group), error bars indicate S.D. Different letters above bars indicate significant differences ($P < 0.05$).

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

Target gene	Primer sequence Forward (5′→3′)	Primer sequence Reverse (5′→3′)	Temperature(°C)	Accession number
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59.3	JQ246442.1
β-defensin-1	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58.4	KT445868
LEAP-2A	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59.3	FJ390414
LEAP-2B	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59.3	KT625603
Mncin2	GAGTTCCCAACCCAACACAT	AAAGGTCTACACAATCTGCCC	60.4	KT625602
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	57.1	JQ692172
IL-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	62.3	KC535507.1
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	60.3	JN663841
IL-12p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55.4	KF944667.1
IL-12p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	59.0	KF944668.1
IL-15	CCTTCCAACAATCTCGCTTC	AACACATCTTCCAGTTCTCCTT	61.4	KT445872
IL-17D	GTGTCCAGGAGAGCACCAAG	GCGAGAGGCTGAGGAAGTTT	62.3	KF245426.1
TNF-α	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC	58.4	HQ696609
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	60.4	JX657682
IL-4/13A	CTACTGCTCGCTTTCGCTGT	CCCAGTTTTCAGTTCTCTCAGG	55.9	KT445871
IL-4/13B	TGTGAACCAGACCCTACATAACC	TTCAGGACCTTTGCTGCTTG	55.9	KT625600
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	61.4	HQ388294
IL-11	GGTTCAAGTCTCTTCCAGCGAT	TGCGTGTTATTTTGTTCAGCCA	57.0	KT445870
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
4E-BP2	CACTTTATTCTCCACCACCCC	TTCATTGAGGATGTTCTTGCC	60.3	KT757306
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	61.4	M25013

[a] LEAP-2, liver expressed-antimicrobial peptide 2; IL, interleukin; TNF-α, tumour necrosis factor α; IFN-γ2, interferon γ2; TGF-β, transforming growth factor β; NF-κB, nuclear factor kappa B; IκBα, inhibitor of κBα; IKK, IκB kinase; TOR, target of rapamycin; S6K1, ribosomal protein S6 kinases 1; 4E-BP, eIF4E-binding proteins.

2.8. Western blot analysis

The protein homogenate and antibodies preparation and the western blotting were conducted according to Jiang et al. [63] and Varady et al. [64]. First, a bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology Inc., China) was used to determine the protein concentrations. Then, the sample proteins (40 μg of protein per lane) were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The target proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 1.5 h at room temperature (RT), the membrane was incubated with primary antibodies at 4 °C overnight according to Fontagné-Dicharry et al. [65]. The same primary antibodies for NF-κBp65 (AF5006, 1:750 dilution), total TOR (AF6308, 1:1000 dilution), phosphorylation of TOR on residue Ser2448 (p-TOR Ser2448) (AF3308, 1:1000 dilution), Lamin B1 (AF5161, 1:1000 dilution) and β-actin (AF7018, 1:3000 dilution) used in our previous study were used in the present study [66]. In grass carp, gcIκBα was identified to be an IκBα homologue [67]. The primary antibody for IκBα (anti-gcIκBα Ab, 1:600 dilution) was kindly provided by the School of Life Science and Technology, University of Electronic Science and Technology of China. After the incubation of primary antibodies, the PVDF membrane was then incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (A0208, 1:8000 dilution, Beyotime Biotechnology Inc., China) for 1.5 h. Immune complexes were visualised using electrochem-iluminescence (ECL) kits (Beyotime Biotechnology Inc., Jiangsu, China). The protein bands were quantified using ImageJ (NIH, USA). The protein levels were expressed as relative values to the control group.

2.9. Calculations and statistical analysis

Growth performance parameters, including percentage weight gain (PWG), feed efficiency (FE), specific growth rate (SGR) and intestinal length index (ILI) were calculated according to the following formulas:

$$\text{PWG (\%)} = 100 \times [\text{final weight (g fish}^{-1}\text{)-initial weight (g fish}^{-1}\text{)}]/\text{initial weight (g fish}^{-1}\text{)};$$

$$\text{SGR (\%)} = 100 \times [\ln[\text{final weight (g fish}^{-1}\text{)}]-\ln[\text{initial weight (g fish}^{-1}\text{)}]]/\text{days};$$

$$\text{FE (\%)} = 100 \times [\text{final weight (g fish}^{-1}\text{)- initial weight (g fish}^{-1}\text{)}]/\text{FI};$$

$$\text{ILI (\%)} = 100 \times [\text{intestine length (cm)/total body length (cm)}];$$

The results are presented as the mean ± standard deviation (SD). All data were subjected to one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range test to determine significant differences at $P < 0.05$ among different treatments with SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Pearson correlation coefficient analysis was conducted using the Bivariate Correlation program in SPSS 19.0. The broken-line analysis was used to estimate the maximum tolerance levels of PA in fish according to Wang et al. [13].

3. Results

3.1. Effects of PA on the growth performance and intestinal growth of on-growing grass carp

The effects of PA on the growth performance and intestinal growth of on-growing grass carp are presented in Table 3. The IBW of fish had no significant difference among all groups ($P > 0.05$). Compared with the control group, the PWG, SGR, FI and FE of fish were all significantly lower ($P < 0.05$) when the PA level increased to 3.2%. The IL was not significantly different among all groups ($P > 0.05$). The ILI of fish was

Table 3
Effects of phytic acid on growth performance and intestinal performance-related parameters of on-growing grass carp.

	Dietary phytic acid levels (%)					
	0	0.8	1.6	2.4	3.2	4.0
IBW[a]	119.78 ± 1.02[a]	119.55 ± 0.39[a]	120.22 ± 0.77[a]	120.67 ± 0.67[a]	120.89 ± 0.77[a]	120.44 ± 1.02[a]
FBW[a]	452.00 ± 25.62[c]	448.22 ± 20.89[c]	439.78 ± 11.48[c]	424.67 ± 19.64[c]	353.56 ± 6.41[b]	289.56 ± 5.67[a]
PWG[a]	277.27 ± 18.53[c]	274.93 ± 17.82[c]	265.84 ± 11.16[c]	251.90 ± 15.06[c]	192.49 ± 6.86[b]	140.41 ± 4.39[a]
SGR[a]	2.21 ± 0.08[c]	2.20 ± 0.08[c]	2.16 ± 0.05[c]	2.10 ± 0.07[c]	1.79 ± 0.04[b]	1.46 ± 0.03[a]
FI[a]	430.77 ± 0.23[e]	430.54 ± 0.14[de]	430.40 ± 0.12[d]	424.03 ± 0.31[c]	351.87 ± 0.12[b]	275.58 ± 0.11[a]
FE[a]	77.12 ± 5.73[c]	76.34 ± 4.85[c]	74.25 ± 2.78[c]	71.69 ± 4.48[bc]	66.12 ± 2.01[ab]	61.36 ± 1.94[a]
IL[b]	41.22 ± 2.06[a]	40.26 ± 2.64[a]	39.19 ± 4.21[a]	40.77 ± 4.44[a]	40.83 ± 3.75[a]	40.57 ± 2.02[a]
ILI[b]	121.65 ± 4.95[a]	119.58 ± 6.33[a]	118.47 ± 10.50[a]	122.10 ± 10.14[a]	132.26 ± 10.45[b]	138.50 ± 5.93[b]
Regression						
Y_{PWG} = -70.2584x + 420.0695; X_{max} = 2.17					R^2 = 0.9720	$P < 0.01$
Y_{SGR} = -0.3992x + 3.0610; X_{max} = 2.24					R^2 = 0.9786	$P < 0.01$
Y_{FE} = -6.6173x + 87.6556; X_{max} = 1.93					R^2 = 0.7694	$P < 0.01$

[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 = 12), and different superscripts in the same row are significantly different ($P < 0.05$). IL, intestine length (cm); ILI, intestine length index (%).

elevated significantly with increased PA levels up to 3.2% compared with the control group ($P < 0.05$).

3.2. Histological examination of the intestine of on-growing grass carp fed graded levels of PA

The histological results of three fish intestinal segments (Fig. 2) showed that compared with the control group, 2.4 and 4.0% PA led to obvious symptoms including lamina propria thickening, increased leukocyte infiltration, nuclear migration, blood capillary hyperemia, epithelial necrosis and epithelial separation from lamina propria in the intestine of on-growing grass carp. The scoring results of intestinal morphological changes are presented in Table 4. Compared with the control group, 2.4 and 4.0% PA significantly aggravated intestinal morphological changes in on-growing grass carp ($P < 0.05$).

3.3. Effects of PA on the enteritis morbidity of on-growing grass carp

The survival rate of all groups in the challenge trial was 100% (unpublished data). The enteritis symptoms of on-growing grass carp are shown in Fig. 3. Compared with the control group, a PA level ≥2.4% caused obvious symptoms of hyperaemia and swelling in fish intestines. The enteritis morbidity of on-growing grass carp after infection, which was based on scoring the noticeable hypertrophy and hyperemia of intestines, is shown in Fig. 3. The enteritis morbidity increased significantly when the PA level increased to 2.4% compared with the control group ($P < 0.05$). Based on the broken-line analysis of fish enteritis morbidity, the maximum tolerance level of PA in a diet for on-growing grass carp was estimated to be 1.68%.

3.4. Effects of PA on the intestinal immune parameters of on-growing grass carp

The activities of the LZ and ACP and the contents of C3, C4 and IgM in the three intestinal segments of on-growing grass carp are presented in Table 5. There were no significant differences of these immune related parameters in fish intestines among the first three groups ($P > 0.05$). When the PA level went up to 2.4%, the activity of LZ and IgM contents significantly decreased in the three intestinal segments compared with the control group ($P < 0.05$). The C3 and C4 contents declined significantly in PI and MI when the PA level reached 2.4%, and in the DI when the PA level increased up to 2.4 and 3.2%, respectively ($P < 0.05$). The activity of ACP was enhanced significantly as the level of PA increased up to 4.0% in the PI and 3.2% in the MI and DI ($P < 0.05$).

3.5. Effects of PA on the intestinal gene expression in on-growing grass carp

3.5.1. Effects of PA on the relative mRNA levels of intestinal antimicrobial peptides in on-growing grass carp

The relative mRNA levels of antimicrobial peptides in the three intestinal segments of on-growing grass carp fed graded levels of PA are shown in Fig. 4. In the PI, MI and DI, the mRNA levels of hepcidin, LEAP-2A, LEAP-2B and β-defensin-1 were all downregulated as PA levels increased ($P < 0.05$). However, Mucin2 mRNA levels in the PI, MI and DI of fish were upregulated by increasing PA levels ($P < 0.05$).

3.5.2. Effects of PA on the relative mRNA levels of intestinal cytokines in on-growing grass carp

In the three fish intestinal segments, the mRNA levels of pro-inflammatory cytokines including TNF-α, IL-1β (except in the DI), IFN-γ2, IL-8, IL-12p40, IL-15 (except in the DI) and IL-17D were upregulated ($P < 0.05$), whereas the mRNA levels of anti-inflammatory cytokines including TGF-β1, IL-4/13A, IL-4/13B, IL-10 and IL-11 were downregulated by increasing PA levels ($P < 0.05$). The gene expression levels of IL-12p35 and TGF-β2 did not show any significant difference among treatments ($P > 0.05$), whereas the mRNA levels of IL-6 were downregulated in the three fish intestinal segments by increasing PA levels ($P < 0.05$).

3.5.3. Effects of PA on the relative mRNA levels of immune-related signalling molecules in on-growing grass carp intestines

The effects of PA on immune-related signalling molecules in the intestine of on-growing grass carp are presented in Fig. 4. With PA levels increasing, the mRNA levels of NF-κBp65, c-Rel, IKKβ, IKKγ, 4E-BP1 and 4E-BP2 were all upregulated ($P < 0.05$), while the mRNA levels of IkBα, TOR and S6K1 were downregulated in the PI, MI and DI of fish ($P < 0.05$). Further, PA had no effects on the mRNA levels of NF-κBp52 and IKKα in the three segments of fish intestine ($P > 0.05$).

3.6. Effects of PA on the NF-κBp65, IκBα and TOR protein levels in the intestine of on-growing grass carp

The protein levels of NF-κBp65, IκBα, p-TOR Ser2448 and T-TOR in the PI, MI and DI of grass carp fed graded levels of PA are shown in Fig. 5 and Fig. 6. In comparison with the control group, the nuclear protein levels of NF-κBp65 in the PI, MI and DI were significantly increased as the PA level increased up to 2.4% ($P < 0.05$). Conversely,

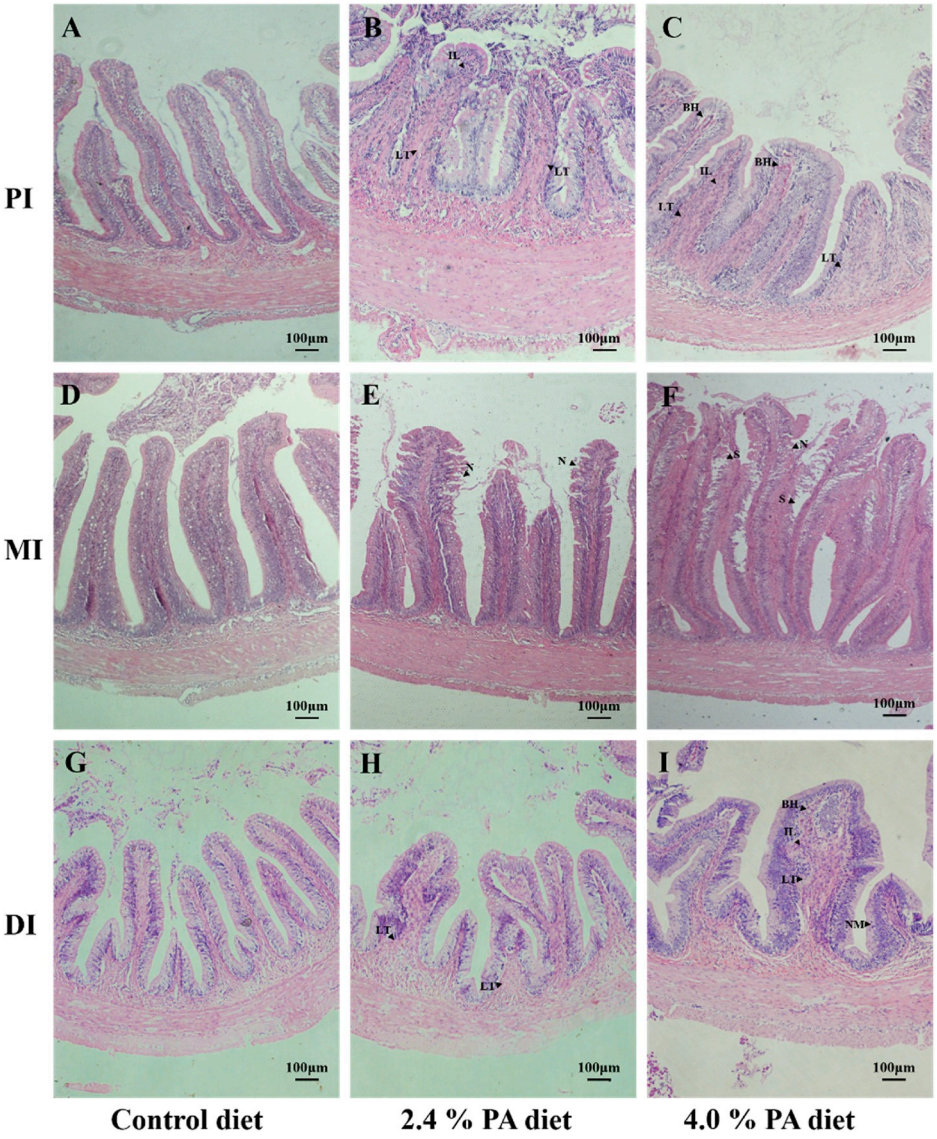

Fig. 2. The intestinal histology of on-growing grass carp fed different levels of phytic acid. (A) control, (B) 2.4% diet and (C) 4.0% diet in PI; (D) control, (E) 2.4% diet and (F) 4.0% diet in MI; (G) control, (H) 2.4% diet and (I) 4.0% diet in DI. The symptoms include lamina propria thickening (LT), increased leukocyte infiltration (IL), nuclear migration (NM), blood capillary hyperemia (BH), epithelial necrosis (N) and epithelial separation from lamina propria (S). The sections were stained with haematoxylin and eosin (H&E) and observed at 100× original magnification.

compared with the control group, the protein levels of IκBα in the PI, MI and DI significantly decreased when the PA levels reached 2.4, 3.2 and 2.4%, respectively ($P < 0.05$). The protein levels of p-TOR and T-TOR in the three intestinal segments significantly decreased as the PA levels increased to 2.4 and 3.2%, respectively ($P < 0.05$).

3.7. Correlation analysis of the intestinal parameters of on-growing grass carp

Correlation analysis (Table 6) indicated that the mRNA levels of upregulated pro-inflammatory cytokines in the corresponding fish intestinal segments were positively correlated with the nuclear NF-κBp65 protein levels. Inverse correlations were found between the protein levels of IκBα and NF-κB, and between the mRNA levels of IKK [β and γ (not α)] and IκBα in the intestines of fish. In addition, the influenced anti-inflammatory cytokines were positively correlated with the protein levels of p-TOR Ser2448 and S6K1 gene expression in the corresponding intestinal segments. The mRNA levels of 4E-BP1 and 4E-BP2 showed negative correlations with p-TOR Ser2448 protein levels and anti-inflammatory cytokines (except TGF-β2) in the three intestinal segments of fish (Table 6).

4. Discussion and summary

4.1. PA caused growth retardation and reduced enteritis resistance of fish

In the present study, we observed that compared with the control group, a PA level ≥3.2% led to poor growth performance (lower PWG, FI and FE) of on-growing grass carp. Based on the broken-line analysis of PWG and FE, the maximum tolerance levels of PA in the diet were estimated to be 2.17 and 1.93% of the diet.

Additionally, as for the histopathological examination of intestines, symptoms such as lamina propria thickening [68], increased leukocyte infiltration [9], blood capillary hyperemia [69], nuclear migration [70] epithelial necrosis [71] and epithelial separation from lamina propria [72] have been confirmed reflecting histopathological changes in fish enteropathy. Our tissue slice results suggest that PA (2.4 and 4.0%) caused fish enteropathy and disrupted fish intestinal mucosal health,

Table 4
Intestinal morphological changes in on-growing grass carp fed diets containing different levels of phytic acid.[a]

Morphology	Dietary phytic acid levels (%)		
	Control (0)	2.4%	4.0%
PI			
Lamina propria thickening	0.67	1.00	1.33
Increased leukocyte infiltration	0.00	1.33	1.33
Nuclear migration	0.00	1.00	1.67
Blood capillary hyperemia	0.00	0.67	0.67
Epithelial necrosis	0.00	0.67	0.33
Epithelial separation from lamina propria	0.00	0.33	0.33
Column totals	0.67[a]	5.00[b]	5.67[b]
MI			
Lamina propria thickening	0.67	1.00	1.67
Increased leukocyte infiltration	0.00	0.67	1.00
Nuclear migration	0.67	1.33	1.33
Blood capillary hyperemia	0.00	0.33	0.33
Epithelial necrosis	0.00	0.67	1.67
Epithelial separation from lamina propria	0.33	1.00	1.33
Column totals	1.67[a]	5.00[b]	7.33[b]
DI			
Lamina propria thickening	0.33	1.33	2.00
Increased leukocyte infiltration	0.00	0.67	0.67
Nuclear migration	0.00	1.00	1.33
Blood capillary hyperemia	0.33	0.67	1.00
Epithelial necrosis	0.00	0.00	0.33
Epithelial separation from lamina propria	0.00	0.67	0.67
Column totals	0.67[a]	4.33[b]	6.00[b]

[a] The morphological changes are based on the microscopy evaluation of 10 micrographs from each fish in each treatment group. Tissue changes were assessed as follows: 0 = not observed; 1 = low frequency (1–3 out of 10 images); 2 = moderate frequency (4–6 out of 10 images) and 3 = high frequency (≥7 out of 10 images); Means, n = 3 fish in each treatment. Different superscript letters among groups mean significant differences.

which could further reduce fish resistance to potential pathogens in the environment [73].

The intestinal health status of fish could be partially reflected by the enteritis resistance [74]. For the first time, we found that a PA level ≥2.4% reduced the fish enteritis resistance ability by increasing enteritis morbidity. The maximum tolerance level of PA based on the analysis of enteritis morbidity (1.68%) was lower than that based on PWG (2.17%), indicating that fish might be more sensitive to PA under the stress of *A. hydrophila*. Furthermore, the intestinal immune function plays a vital role in enteritis resistance, which is partly related to the immune responses in fish [49,75]. Hence we next investigated the effects of PA on fish intestinal immune responses.

4.2. PA impacted innate and adaptive immune components in fish intestine

The immune function of fish is based on innate and adaptive immune components such as ACP, LZ, complements (C3 and C4), antimicrobial peptides and immunoglobulins [76,77]. The results displayed that a PA level ≥2.4% suppressed fish intestinal bactericidal activity, which is partly associated with decreasing most immune components.

Intriguingly, different trends were found in ACP activity and Mucin2 gene expression in the three fish intestinal segments. The possible reasons are as follows. First, the elevation of ACP activity is possibly related to inducible nitric oxide synthase (iNOS). It was reported that PA suppressed iNOS activity in the brains of mice [78], which could reduce nitric oxide (NO) generation in rat intestines [79]. A lower level of NO activated ACP in RAW-264.7 macrophages [80]. These results support our hypothesis. Second, the upregulation of Mucin2 mRNA levels by PA might be partly relevant to IL-6. According to Shan et al. [81], a decreased secretion of IL-6 led to an upregulation of Mucin2 gene expression in a mice colon model. Our data displayed that PA downregulated the mRNA levels of IL-6 in the three segments of intestine, which is consistent with our hypothesis. However, these hypotheses need further investigation to be verified.

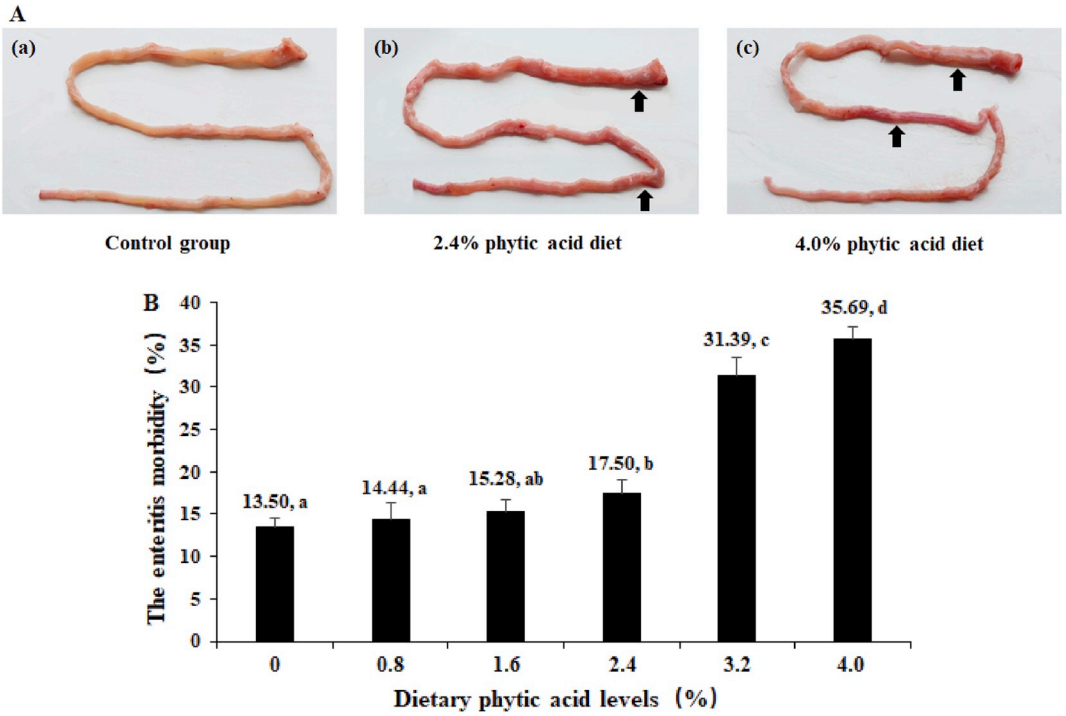

Fig. 3. The enteritis symptoms (A) and morbidity (B) of on-growing grass carp fed graded levels of phytic acid after infection with *Aeromonas hydrophila*. (A) Compared with the control group, PA (2.4% and 4.0%) caused obvious enteritis symptoms including red intestinal coloration and swelling (arrows). (B) Data represent means (n = 24 individuals in each group), error bars indicate S.D. Values above bars with different letters are significantly different ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
The immune related parameters in the PI, MI and DI of on-growing grass carp fed graded levels of phytic acid.[a]

	Dietary phytic acid levels (%)					
	0	0.8	1.6	2.4	3.2	4.0
PI						
LZ	255.79 ± 13.51^{c}	255.99 ± 21.39^{c}	239.85 ± 17.94^{c}	209.16 ± 19.78^{b}	182.33 ± 10.31^{a}	163.30 ± 15.34^{a}
ACP	223.49 ± 9.68^{a}	227.60 ± 10.04^{ab}	226.68 ± 18.32^{a}	234.30 ± 12.75^{ab}	238.14 ± 18.97^{ab}	246.23 ± 15.40^{b}
C3	30.15 ± 1.72^{c}	28.23 ± 2.39^{c}	28.80 ± 3.38^{c}	24.85 ± 2.09^{b}	23.07 ± 2.42^{ab}	20.90 ± 1.84^{a}
C4	8.80 ± 0.61^{c}	8.76 ± 0.48^{c}	8.33 ± 0.76^{c}	7.31 ± 0.55^{b}	6.64 ± 0.52^{b}	5.68 ± 0.55^{a}
IgM	74.81 ± 6.75^{d}	74.62 ± 5.18^{d}	73.29 ± 8.70^{cd}	66.49 ± 3.83^{bc}	62.69 ± 6.63^{b}	54.63 ± 5.03^{a}
MI						
LZ	296.16 ± 9.35^{d}	292.29 ± 13.15^{d}	285.03 ± 18.05^{cd}	270.98 ± 11.54^{c}	255.56 ± 8.43^{b}	218.70 ± 8.21^{a}
ACP	296.70 ± 12.48^{a}	304.52 ± 13.70^{a}	304.87 ± 21.16^{a}	307.81 ± 13.92^{ab}	327.35 ± 15.76^{bc}	331.92 ± 22.91^{c}
C3	33.06 ± 1.86^{c}	32.54 ± 1.27^{c}	32.04 ± 2.49^{bc}	29.88 ± 1.89^{b}	25.72 ± 1.26^{a}	24.54 ± 2.91^{a}
C4	14.13 ± 1.45^{d}	14.13 ± 0.60^{d}	13.83 ± 1.11^{d}	12.64 ± 0.67^{c}	10.49 ± 1.16^{b}	8.88 ± 0.77^{a}
IgM	79.34 ± 4.38^{c}	76.31 ± 6.01^{c}	77.48 ± 7.02^{c}	68.28 ± 3.05^{b}	63.30 ± 5.08^{ab}	60.38 ± 5.69^{a}
DI						
LZ	322.46 ± 26.87^{c}	315.11 ± 14.66^{c}	323.18 ± 37.18^{c}	271.70 ± 23.80^{b}	258.74 ± 15.74^{b}	222.63 ± 15.34^{a}
ACP	287.63 ± 13.39^{a}	296.22 ± 12.41^{a}	292.04 ± 12.00^{a}	299.12 ± 15.29^{a}	316.36 ± 17.70^{b}	329.02 ± 9.61^{b}
C3	44.90 ± 3.19^{c}	43.67 ± 4.25^{c}	43.71 ± 4.02^{c}	38.15 ± 2.05^{b}	36.21 ± 2.28^{b}	29.70 ± 1.98^{a}
C4	16.07 ± 1.44^{b}	15.98 ± 0.70^{b}	15.51 ± 1.16^{b}	15.26 ± 0.75^{b}	12.26 ± 1.26^{a}	11.17 ± 0.30^{a}
IgM	104.58 ± 6.54^{d}	105.05 ± 9.75^{d}	103.13 ± 6.85^{cd}	95.44 ± 4.79^{bc}	90.13 ± 6.86^{b}	75.77 ± 4.91^{a}

[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).

Studies concerning the effects of ANFs on fish intestinal immune components are scarce. Our previous study confirmed that gossypol in the diet could reduce the contents or activities of intestinal immune components in grass carp [13]. Similar phenomena were discovered in the present study except for increasing ACP activity in fish intestines. Meanwhile, the two ANFs influenced fish intestinal mRNA levels of hepcidin, LEAP-2A, LEAP-2B and Mucin2 in different patterns. The reasons for the different effects are unknown and need more investigation. Additionally, the intestinal mucosal immune status of fish is closely related to inflammatory responses, which are primarily mediated by cytokines [82]. Hence, we next examined the effects of PA on the mRNA levels of intestinal cytokines in on-growing grass carp.

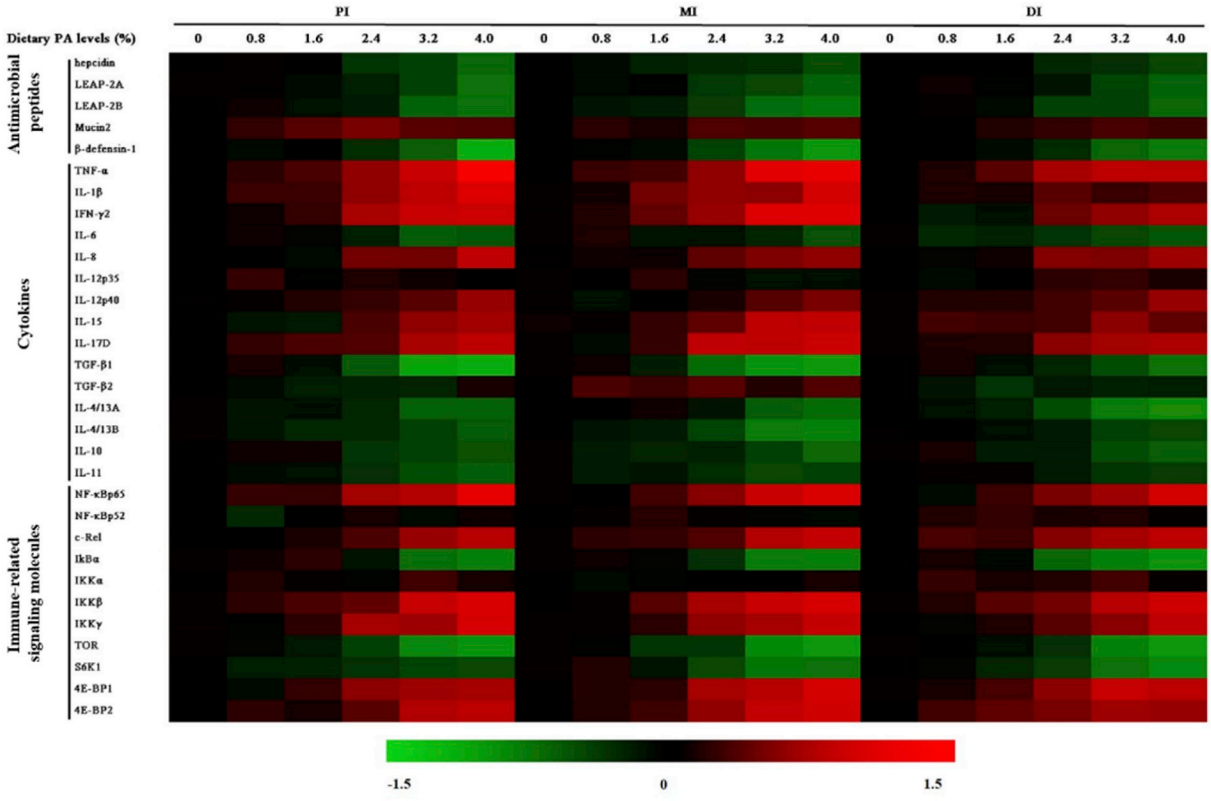

Fig. 4. Heat-map of the mRNA levels of antimicrobial peptides, cytokines, NF-κB signalling pathway-related molecules and TOR signalling pathway-related molecules in the PI, MI and DI of on-growing grass carp fed graded levels of phytic acid. The values of upregulation (red) and downregulation (green) (i.e. from 1.5 to −1.5) represent log2 fold changes. N = 6 for each phytic acid level. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

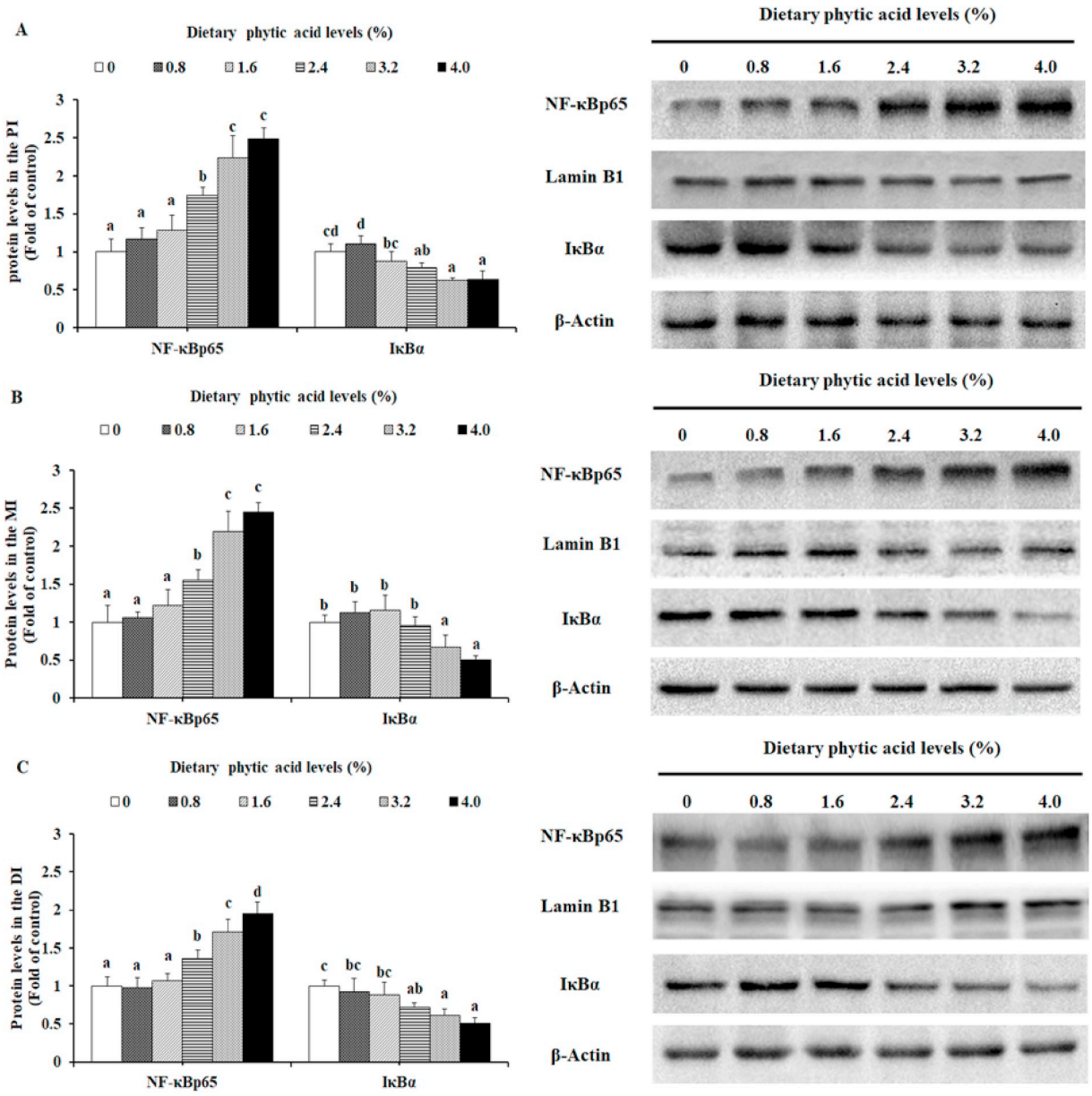

Fig. 5. Western blot analysis of NF-κBp65 and IκBα proteins in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means (n = 3 replicates in each group), error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$).

4.3. PA aggravated fish intestinal inflammatory responses and its possible mechanisms

Inflammation is a stereotypical host defence mechanism that protects the host from pathogen infection, but undue self-regulated inflammatory responses can disrupt the balance of immune functions, contributing to the perpetuation or progression of disease in humans [28]. It was reported that the aggravation of undue inflammation is associated with the upregulation of pro-inflammatory cytokines and the downregulation of anti-inflammatory cytokines in fish [83], which are mainly regulated by the NF-κB and mTOR signalling pathways in mammals, respectively [84,85]. In addition, it is worth mentioning that the degradation of IκBα protein and the increase of nuclear NF-κBp65 protein levels are considered to be a hallmark of NF-κB nuclear translocation and NF-κB signalling pathway activation in mammals [86,87]. The phosphorylation of TOR on Ser2448 is considered to be a hallmark of the activation of the TOR signalling pathway in rainbow trout (*Oncorhynchus mykiss*) [88]. In the present study, increasing PA levels aggravated fish intestinal inflammation associated with upregulating most pro-inflammatory cytokine mRNA levels and downregulating most anti-inflammatory cytokine mRNA levels. Furthermore, the correlation analysis results indicate that the regulation of fish intestinal cytokine mRNA levels might be partially related to activating the NF-κB signalling pathway and inhibiting the TOR signalling pathway.

The potential reasons for some distinctive cytokine gene expressions in the three intestinal segments are discussed as follows. First, the upregulation of fish intestinal IL-12p40 (rather than IL-12p35) mRNA levels in is likely to be relevant to TNF-α. In our study, certain levels of PA upregulated TNF-α mRNA levels in all segments of fish intestine. Wang and Husain [89] demonstrated that TNF-α could only upregulate the IL-12p40 (rather than IL-12p35) gene expression in Atlantic salmon HK cells, which supports our speculation. Second, IL-15 mRNA levels were only upregulated in the PI and MI (not DI), which might be associated with IL-1β. It has been reported that the elevation of IL-1β gene expression could upregulate IL-15 mRNA level in rat pancreatic islet cells [90]. The present study showed that PA could only upregulate

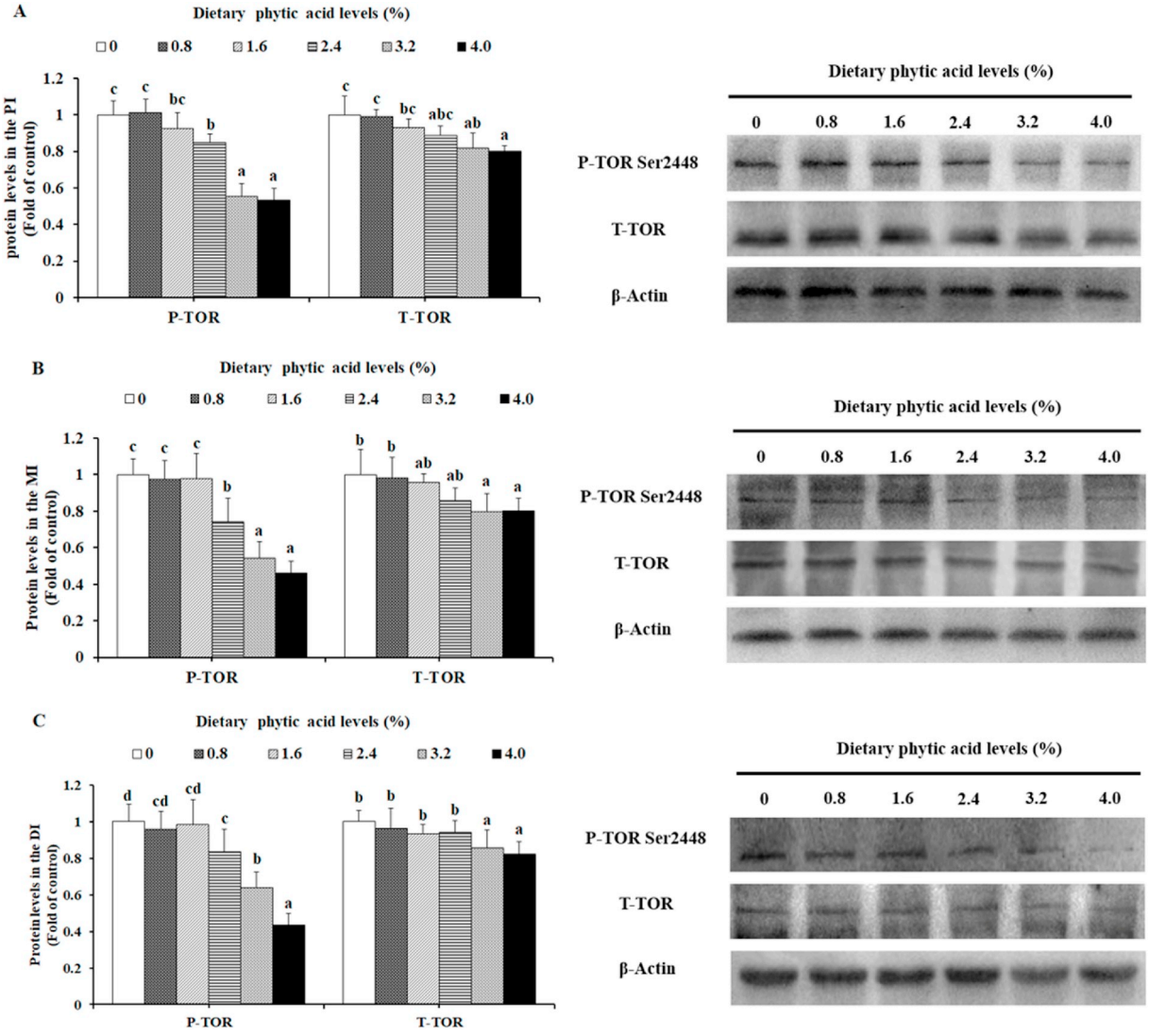

Fig. 6. Western blot analysis of TOR phosphorylation at Ser2448 and T-TOR proteins in the PI (A), MI (B) and DI (C) of on-growing grass carp. Data represent means (n = 3 replicates in each group), error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$).

IL-1β mRNA levels in the PI and MI (rather than DI) of grass carp, which may partially explain the upregulation model of IL-15 in fish intestines. Third, PA upregulated IL-1β mRNA levels only in the PI and MI (not DI), which might be partly related to increasing β-glucosidase activity. Okazaki and Katayama [91] demonstrated that PA elevated faecal β-glucosidase activity in rats, which could promote IL-1β gene expression in the peripheral blood of humans [92]. Further, β-glucosidase activities were only sustained at high levels in the proximal and mid (but not dismal) small intestines in human foetuses [93]. These results support our assumption. Fourth, unlike TGF-β1, no significant changes were found in fish intestinal TGF-β2 mRNA levels. The reason might be associated with glucose. Kumar et al. [94] demonstrated that PA could lower the blood glucose level in Nile tilapia (*Oreochromis niloticus* L.). In human mesangial cells, decreasing glucose only downregulated TGF-β1 (rather than TGF-β2) gene expression [95], which is consistent with our hypothesis. Finally, the reason for PA downregulating fish intestinal IL-6 mRNA levels might be partially explained by butyrate. Okazaki and Katayama [91] founded that PA could promote the production of butyrate in the gut of rats. Our previous study confirmed that butyrate downregulated IL-6 gene expression in the three intestinal segments of grass carp [96], which supports our hypothesis. However, more evidence is required to verify these assumptions.

In addition, the potential reasons for the unaffected mRNA levels of NF-κBp52 and IKKα are explained as follows. First, the unaffected NF-κBp52 mRNA levels might be relevant to IKKα. IKKα is the predominant stimulus for processing p100 to p52 in humans [97,98]. The present study found that PA had no impact on fish intestinal IKKα mRNA levels, which supports our hypothesis. Second, PA upregulated IKKβ and IKKγ (not IKKα) mRNA levels in fish intestines might be associated with IFN-γ. The present study found that PA upregulated fish intestinal IFN-γ mRNA levels. In U937 cells, IFN-γ elevated protein kinase Cζ (PKC-ζ) activity [99], which could only upregulate the mRNA levels of IKKβ and IKKγ (not IKKα) in Kupffer cells [100]. Therefore, we assume that PA might upregulate IFN-γ to activate PKC-ζ, resulting in the upregulation of IKKβ and IKKγ (rather than IKKα) in fish intestines. However, these suppositions should be confirmed through further investigation.

Several studies have previously focused on the effects of ANFs on fish intestinal inflammatory responses. ANFs including soybean β-conglycinin [10], soybean glycinin [101] and gossypol [13] all aggravated fish intestinal inflammation. Similar results were found in the present study. However, the patterns of the pro-inflammatory effects vary among ANFs. We compared the pro-inflammatory effects of gossypol with PA and found the following interesting phenomena. First, the mRNA levels of intestinal IL-12, IL-4 and TGF-β were regulated in different genotype patterns between fish fed gossypol and those fed PA. Second, the mRNA levels of IL-1β, IL-10 and c-Rel were regulated in

Table 6
Correlation analysis of parameters in the intestine of on-growing grass carp.

Independent parameters	Dependent parameters	PI		MI		DI	
		Correlation coefficients	*P*	Correlation coefficients	*P*	Correlation coefficients	*P*
NF-κBp65 protein level	TNF-α	+0.962	< 0.01	+0.995	< 0.01	+0.918	< 0.05
	IFN-γ2	+0.907	< 0.05	+0.987	< 0.01	+0.972	< 0.01
	IL-1β	+0.993	< 0.01	+0.889	< 0.05	+0.712	= 0.112
	IL-8	+0.950	< 0.01	+0.975	< 0.01	+0.937	< 0.01
	IL-12p40	+0.955	< 0.01	+0.975	< 0.01	+0.946	< 0.01
	IL-15	+0.956	< 0.01	+0.996	< 0.01	+0.732	= 0.098
	IL-17D	+0.966	< 0.01	+0.896	< 0.05	+0.957	< 0.01
	IκBα protein level	−0.927	< 0.01	−0.950	< 0.01	−0.979	< 0.01
p-TOR protein level	TGF-β1	+0.966	< 0.01	+0.945	< 0.01	+0.949	< 0.01
	IL-4/13A	+0.954	< 0.01	+0.981	< 0.01	+0.924	< 0.01
	IL-4/13B	+0.877	< 0.05	+0.973	< 0.01	+0.939	< 0.01
	IL-10	+0.898	< 0.05	+0.828	< 0.05	+0.887	< 0.05
	IL-11	+0.957	< 0.01	+0.883	< 0.05	+0.951	< 0.01
	S6K1	+0.868	< 0.05	+0.930	< 0.01	+0.935	< 0.01
	4E-BP1	−0.912	< 0.05	−0.958	< 0.01	−0.898	< 0.05
	4E-BP2	−0.977	< 0.01	−0.991	< 0.01	−0.841	< 0.05
NF-κBp65	TNF-α	+0.981	< 0.01	+0.983	< 0.01	+0.914	< 0.05
	IFN-γ2	+0.879	< 0.05	+0.990	< 0.01	+0.949	< 0.01
	IL-1β	+0.988	< 0.01	+0.911	< 0.05	+0.731	= 0.099
	IL-8	+0.979	< 0.01	+0.964	< 0.01	+0.944	< 0.01
	IL-12p40	+0.975	< 0.01	+0.971	< 0.01	+0.957	< 0.01
	IL-15	+0.921	< 0.01	+0.993	< 0.01	+0.645	= 0.167
	IL-17D	+0.922	< 0.01	+0.925	< 0.01	+0.929	< 0.01
c-Rel	TNF-α	+0.970	< 0.01	+0.992	< 0.01	+0.948	< 0.01
	IFN-γ2	+0.859	< 0.05	+0.970	< 0.01	+0.908	< 0.05
	IL-1β	+0.979	< 0.01	+0.846	< 0.05	+0.842	< 0.05
	IL-8	+0.924	< 0.01	+0.829	< 0.05	+0.968	< 0.01
	IL-12p40	+0.965	< 0.01	+0.955	< 0.01	+0.934	< 0.01
	IL-15	+0.967	< 0.01	+0.983	< 0.01	+0.811	< 0.05
	IL-17D	+0.970	< 0.01	+0.821	< 0.05	+0.974	< 0.01
IκBα	NF-κBp65	−0.904	< 0.05	−0.981	< 0.01	−0.896	< 0.05
	c-Rel	−0.968	< 0.01	−0.957	< 0.01	−0.915	< 0.05
IKKβ	IκBα	−0.947	< 0.01	−0.968	< 0.01	−0.972	< 0.01
IKKγ	IκBα	−0.874	< 0.05	−0.953	< 0.01	−0.933	< 0.01
TOR	TGF-β1	+0.977	< 0.01	+0.940	< 0.01	+0.982	< 0.01
	IL-4/13A	+0.973	< 0.01	+0.882	< 0.05	+0.975	< 0.01
	IL-4/13B	+0.938	< 0.01	+0.958	< 0.01	+0.997	< 0.01
	IL-10	+0.924	< 0.01	+0.890	< 0.05	+0.962	< 0.01
	IL-11	+0.996	< 0.01	+0.884	< 0.05	+0.970	< 0.01
	S6K1	+0.945	< 0.01	+0.934	< 0.01	+0.990	< 0.01
	4E-BP1	−0.957	< 0.01	−0.920	< 0.01	−0.981	< 0.01
	4E-BP2	−0.967	< 0.01	−0.959	< 0.01	−0.932	< 0.01

distinctive intestinal segment patterns between fish fed the two ANFs. Third, intestinal IL-6, IL-17 and 4E-BP2 gene expressions showed different trends between fish fed the two ANFs. The reasons for these distinctive phenomena were not clear, and they deserve further investigation.

4.4. The maximum tolerance levels of PA for on-growing grass carp based on different indices

In the present study, PA depressed the growth and intestinal immune function of on-growing grass carp. Based on the broken-line analysis of the PWG, enteritis morbidity, IgM content and LZ activity in the PI (Fig. 7), we estimated the maximum tolerance levels of PA for on-growing grass carp (120.56–452.00 g) to be 2.17, 1.68, 1.47 and 1.18% of the diet, respectively. In addition, a study showed that the maximum tolerance level of PA for juvenile grass carp (22.3–49.6 g) was lower than 0.5% based on the direct analysis of weight gain [102], suggesting that on-growing grass carp are more tolerable to PA than juvenile ones.

4.5. Summary

This study confirmed that PA suppressed the growth performance of on-growing grass carp. Based on our results, we demonstrated for the first time that PA depressed fish intestinal immune function and aggravated enteritis (see Fig. 8). The detailed mechanisms are displayed as follows: PA (1) impaired intestinal immunity by reducing IgM, C3 and C4 contents and LZ activity and downregulating the transcript abundance of antimicrobial peptides including hepcidin, LEAP-2A, LEAP-2B, and β-defensin-1; (2) aggravated the intestinal inflammation responses partly associated with the upregulation of pro-inflammatory cytokines including TNF-α, IFN-γ2, IL-8, IL-12p40, and IL-17D and the downregulation of anti-inflammatory cytokines including TGF-β1, IL-4/13A, IL-4/13B, IL-10 and IL-11 in the three fish intestinal segments, which are partially associated with the NF-κB signalling pathway [(IKKβ, IKKγ but not IKKα)/IκBα/(NF-κBp65, c-Rel but not NF-κBp52)] and the TOR signalling pathway [TOR/(S6K1/4E-BP1 and 4E-BP1)], respectively; and (3) surprisingly increased ACP activity and Mucin2

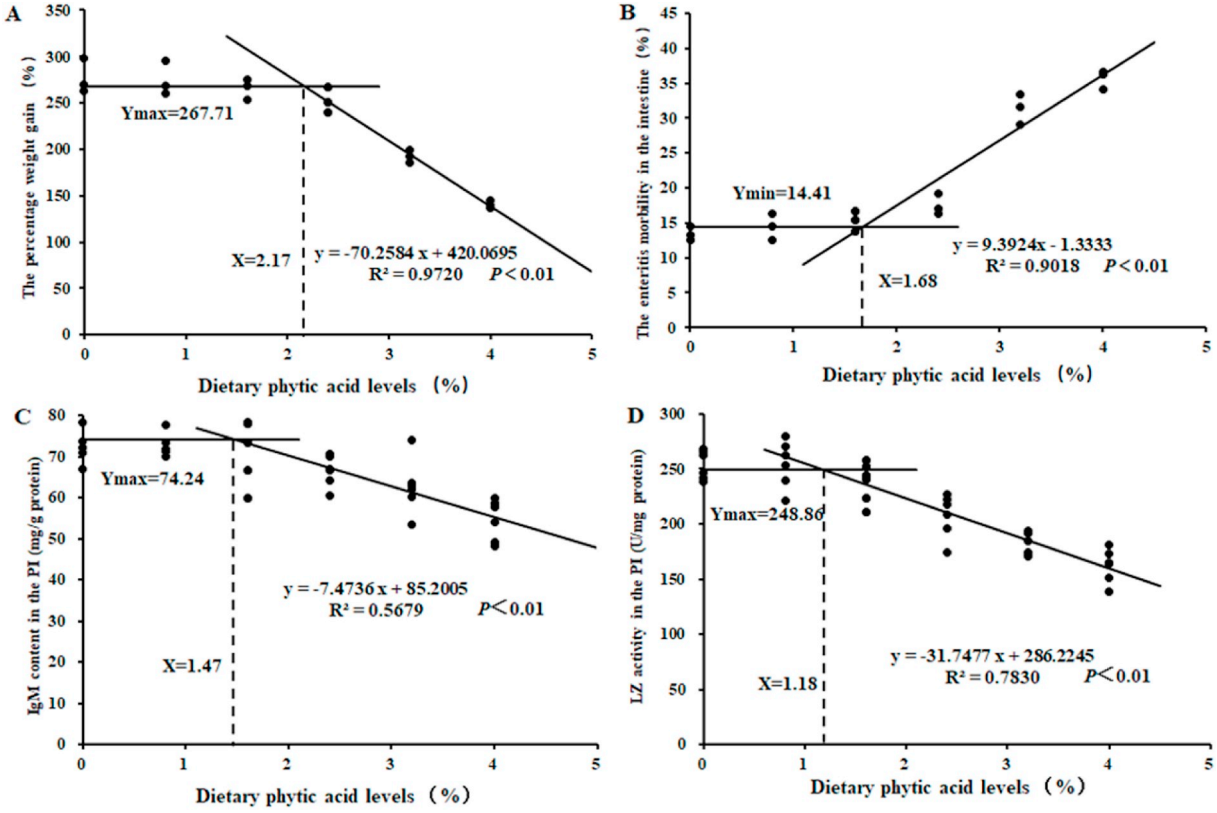

Fig. 7. Broken-line analysis of the PWG (A), enteritis morbidity (B), IgM content (C) and LZ activity (D) in the PI of on-growing grass carp fed graded levels of phytic acid.

gene expressions and downregulated IL-6 gene expression, but had no effects on the mRNA levels of IL-1β (only in the DI), IL-12p35, IL-15 (only in the DI) and TGF-β2 in the three segments of fish intestine. Further, the effects of PA and gossypol on fish intestinal immune status were distinctive, suggesting that different ANFs may impact fish intestinal health through different mechanisms. Based on the PWG, enteritis morbidity and IgM content as well as LZ activity in the PI, the maximum tolerance levels of PA in the diet for on-growing grass carp

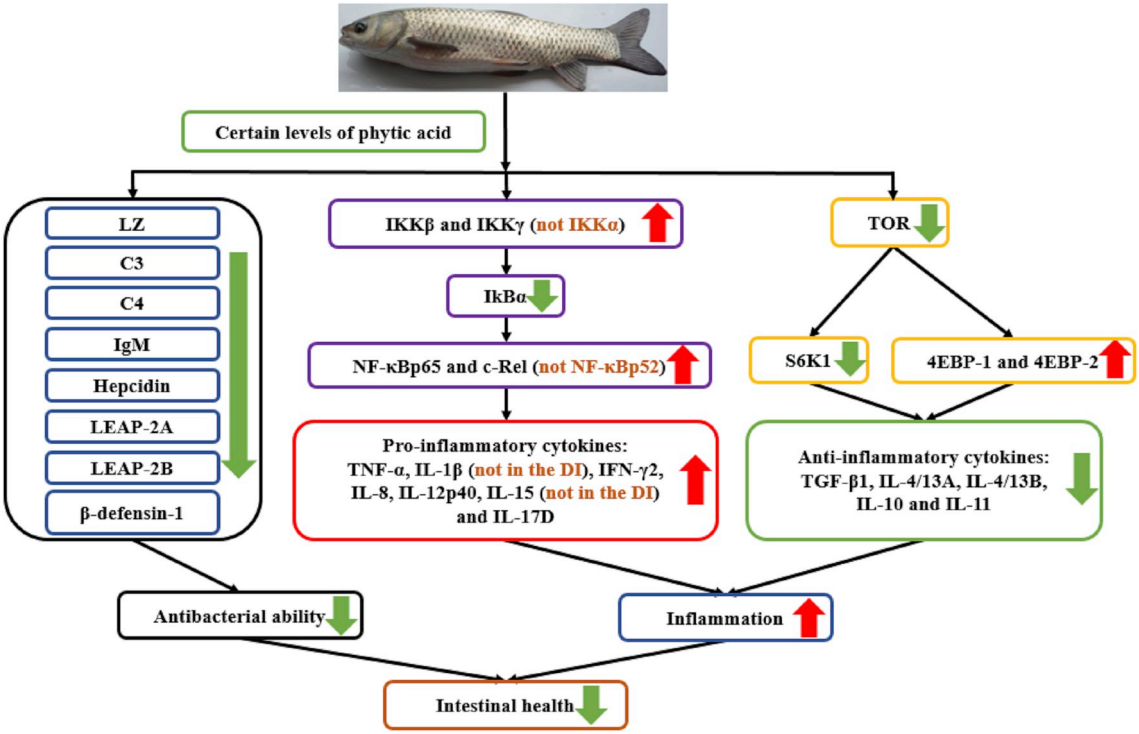

Fig. 8. Potential action pathways of phytic acid impacting intestinal immune status of fish.

were estimated to be 2.17, 1.68, 1.47 and 1.18% of the diet, respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.045.

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