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Dietary gossypol reduced intestinal immunity and aggravated inflammation in on-growing grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

The present study explored the effects of dietary gossypol on the gut health of on-growing grass carp. The fish were fed six diets containing different levels of free gossypol (0, 121.38, 243.94, 363.89, 759.93 and 1162.06 mg/kg diet) from gossypol-acetic acid for 60 days and then challenged with *Aeromonas hydrophila* for 14 days. The results showed that dietary gossypol (1) could aggravate enteritis and damage the structure of intestinal epithelial cells, (2) decreased the lysozyme (LZ) and Acid phosphatase (ACP) activities, complement 3 (C3), C4 and immunoglobulin M (IgM) contents, and it down-regulated the Hepcidin (rather than distal intestine (DI)), immunoglobulin Z (IgZ), liver-expressed antimicrobial peptide (LEAP)-2B, Mucin2 and β-defensin-1 mRNA levels in the proximal intestine (PI), mid intestine (MI) and DI, (3) up-regulated intestinal pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interferon γ2 (IFN-γ2), interleukin 1β (IL-1β), IL-6 (only in PI), IL-8 and IL-12p35 mRNA levels partly related to nuclear factor kappa B (NF-κB) signalling, and (4) down-regulated the mRNA levels of anti-inflammatory cytokines such as transforming growth factor (TGF)-β1, TGF-β2, interleukin 4/13A (IL-4/13A) (except IL-4/13B), IL-10 and IL-11 partly relating to target of rapamycin (TOR) signalling in the intestines of on-growing grass carp. Moreover, the dietary gossypol had no impact on the LEAP-2A, IL-12P40, IL-17D, IL-10, NF-κBp52, IKKα and eIF4E-binding proteins 2 (4E-BP2) mRNA levels in the intestines. Finally, based on the intestinal histopathological results, enteritis morbidity, LZ activity and IgM content, the safe dose of gossypol in the diets for on-growing grass carp should be less than 103.42 mg/kg diet.

1. Introduction

Fish intestine is constantly exposed to various foreign substances, such as feed toxic ingredients, that always weaken intestinal health [1,2]. As we know, intestinal health was closely related to the intestinal structure integrity and the intestinal immune function in fish [3]. Gossypol is known as a toxic compound present in cottonseed meal which is widely used in fish feed [4]. Our previous study observed that dietary gossypol could impair the intestinal structural integrity of young grass carp [5]. However, whether gossypol impaired the intestinal immune function has not yet been studied in animals. In grass carp, dietary gossypol decreased the threonine contents in the intestines [5], while the low levels of threonine could decrease the intestinal immune function [6]. Hence, there might be a relationship between gossypol and intestinal immune function in fish.

In fish, the intestinal immune function is closely related to innate immune components such as lysozyme (LZ), acid phosphatase (ACP), complements (such as C3 and C4) and antimicrobial peptides (such as hepcidin, liver-expressed antimicrobial peptide (LEAP)-2A, LEAP-2B and β-defensin) and adaptive immune components such as immunoglobulin M (IgM) [7,8]. However, there are no studies focused on the effects of gossypol on innate and adaptive immune components in the intestine of animals. A study reported that gossypol could suppress the activity of protein kinase C (PKC) in spermatocyte [9], while the inhibition of PKC may reduce the LZ release in alveolar macrophages [10]. Watkins et al. [11] reported that gossypol could bind iron in the

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bloodstream, causing iron deficiency in broiler chickens. Our previous study demonstrated that iron deficiency reduced C3 and C4 contents in the gill of grass carp [12]. In channel catfish, gossypol binds to lysine in feed and leads to the lysine deficiency [13]. In gilthead sea bream muscle cells, lysine deficiency decreased the insulin-like growth factor (IGF)-1 expression [14]. The inhibition of IGF-1 could suppress the β-defensin expression in human keratinocytes [15]. These data implied that gossypol might affect intestinal immune immunity in animals, and the possibility warrants investigation.

The intestinal immune function is also associated with the cytokines, which include pro-inflammatory cytokines (such as tumor necrosis factor α (TNF-α), IL-1 and IL-6) and anti-inflammatory cytokines (such as interleukin 10 (IL-10) and transforming growth factor β (TGF-β)) [16]. Moreover, studies in human have confirmed that the pro-inflammatory and anti-inflammatory cytokines could be modulated by nuclear factor κB (NF-κB) [17] and target of rapamycin (TOR) [18], respectively. Our previous study indicated that dietary gossypol inhibited the TOR signalling in the intestines of young grass carp [5]. However, there is no report investigating the effects of gossypol on the NF-κB signalling and the inflammatory cytokines in animal intestine. In rat liver, gossypol could reduce the activity of protein kinase A (PKA) [19], while the inhibition of PKA promoted the production of TNF-α in human dendritic cells [20]. Bian et al. [21] found that gossypol could activate the AMP-activated kinase (AMPK) signalling in turbot primary muscle cells. The activation of AMPK could inhibit the TGF-β expression in the human proximal tubular cell [22]. A study in human promyelocytic leukemia cells reported that gossypol suppressed the activity of protein phosphatase 2A (PP2A) [23]. The inhibition of PP2A could activate NF-κBp65 in murine macrophage [24]. Therefore, the above observations indicate that there might be a potential relationship between gossypol and multiple cytokines, as well as NF-κB and TOR signalling in animals, which remains to be elucidated.

In general, on the basis of our previous study that gossypol decreased the growth and impaired the intestinal integrity of fish [5], for the first time, this study was performed to investigate the effect of gossypol on the intestinal immune function and the possible regulatory mechanisms of animals. Studies have shown that the nutrition requirements of fish may vary with different indices [6,25]. Similarly, the safe dose of gossypol for animals should be estimated by using the different indices. Grass carp is the biggest contributor to the world's aquaculture production (FAO, 2015) [26]. Thus, we also explored the safe dose of gossypol in the diets of on-growing grass carp based on multiple indexes, which may provide a reference for dietary gossypol levels on the health of fish.

2. Methods

2.1. Diet and feeding trial

The present study used the identical animal experiment as our previous study [5]. Formulation and proximate composition of the basal diet are presented in Table 1. The experimental diet and the procedures for diet preparation and storage (−20 °C) were the same as our previous study. The basal diet was supplemented with free gossypol from gossypol-acetic acid (Ci Yuan Biotechnology Co., Ltd. Shanxi of China (purity ≥ 98%)) at levels of 0, 125, 250, 375, 775 and 1175 mg/kg diets. The final free gossypol concentrations of the six diets were 0 (control), 121.38, 243.94, 363.89, 759.93 and 1162.06 mg/kg diet, which were determined by high-performance liquid chromatography (HPLC) [27]. After being prepared completely, all the ingredients were thoroughly mixed and extruded as pellets. After drying, the diets were stored at −20 °C until used according to Guardiola et al. [28].

The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. The grass carp used in this study were purchased from Tong Wei hatchery (Sichuan, China). Prior to the experiment, fish were acclimatized to the experimental environment for 4 weeks according to Ji et al. [29]. Then, 540 fish (mean weight 230.93 ± 0.50 g) were randomly assigned to eighteen experimental cages (1.4 L × 1.4 W × 1.4 H m), resulting in 30 fish per cage as described in our laboratory study [30]. Each cage was equipped with a disc of 100 cm diameter in the bottom to collect the uneaten feed, according to our laboratory study [31]. For the feeding trial, fish were fed with their respective diets to apparent satiation four times daily according to Tu et al. [32] for 60 days. Thirty minutes after feeding, the uneaten feed was collected, dried and weighed to calculate the feed intake, as previously described by Tian et al. [33]. During the experiment, the water temperature was averaged at 27 ± 2 °C, and the pH value was maintained at 7.0 ± 0.4. The dissolved oxygen was not less than 6.0 mg O/L. The experimental units were under natural light and dark cycle.

Table 1
Formulation and chemical composition of the experimental diets (%).

Ingredients	1	2	3	4	5	6
Fish meal	4.80	4.80	4.80	4.80	4.80	4.80
Soybean protein concentrate	23.50	23.50	23.50	23.50	23.50	23.50
Rice protein concentrate meal	18.11	18.11	18.11	18.11	18.11	18.11
Corn starch	15.0800	15.0675	15.0550	15.0425	15.0025	14.9625
α-starch	24.00	24.00	24.00	24.00	24.00	24.00
Fish oil	2.88	2.88	2.88	2.88	2.88	2.88
Soybean oil	0.77	0.77	0.77	0.77	0.77	0.77
Microcrystalline cellulose	5.00	5.00	5.00	5.00	5.00	5.00
$Ca(H_2PO4)_2$	1.29	1.29	1.29	1.29	1.29	1.29
Vitamin premix[a]	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[b]	2.00	2.00	2.00	2.00	2.00	2.00
Gossypol	0.00	0.0125	0.0250	0.0375	0.0775	0.1175
DL-Met (99%)	0.47	0.47	0.47	0.47	0.47	0.47
L-Trp (99%)	0.02	0.02	0.02	0.02	0.02	0.02
L-Thr (98.5%)	0.03	0.03	0.03	0.03	0.03	0.03
Choline chloride (50%)	1.00	1.00	1.00	1.00	1.00	1.00
Ethoxyquin (30%)	0.05	0.05	0.05	0.05	0.05	0.05
Chemical composition (%)						
Moisture[c]	12.66	12.58	12.57	12.62	12.58	12.61
Crude protein[c]	29.72	29.62	29.75	29.80	29.65	29.71
Crude lipid[c]	5.60	5.62	5.55	5.58	5.58	5.60
ω-3 Fatty acids[d]	1.04	1.04	1.04	1.04	1.04	1.04
ω-6 Fatty acids[d]	0.96	0.96	0.96	0.96	0.96	0.96
Available phosphorus[e]	0.40	0.40	0.40	0.40	0.40	0.40
Free gossypol (mg/kg)[f]	0.00	121.38	243.94	363.89	759.93	1162.06

[a] Per kilogram of vitamin premix (g/kg): retinyl acetate (500,000 IU/g), 2.10; cholecalciferol (500,000 IU/g), 0.40; DL-a-tocopherol acetate (50%), 12.58; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.09; ascorhyl acetate (95%), 4.31; niacin (99%), 4.04; meso-inositol (98%), 19.39; calcium-D-pantothenate (98%), 3.85; riboflavin (80%), 0.73; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with maize starch to 1 kg.

[b] Per kilogram of mineral premix (g/kg): MnSO4·H2O (31.8% Mn), 2.6590; MgSO4·H2O (15.0% Mg), 200.0000; FeSO4·H2O (30.0% Fe), 12.2500; ZnSO4·H2O (34.5% Zn), 8.2460; CuSO4·5H2O (25.0% Cu), 0.9560; KI (76.9% I), 0.0650; Na2SeO3 (44.7% Se), 0.0168. All ingredients were diluted with maize starch to 1 kg.

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

[d] ω-3 and ω-6 were calculated according to Zeng et al. [90] and calculated according to NRC (2011).

[e] Available phosphorus were calculated according to NRC (2011).

[f] Free gossypol contents were measured values.

Table 2
Real-time PCR primer sequences *.

Target gene	Primer sequence Forward (5′→3′)	Primer sequence Reverse (5′→3′)	Temperature (°C)	Accession number
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59.3	JQ246442.1
LEAP-2A	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59.3	FJ390414
LEAP-2B	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59.3	KT625603
Mucin 2	GAGTTCCCAACCCAACACAT	AAAGGTCTACACAATCTGCCC	60.4	KT625602
β-defensin-1	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58.4	KT445868
IgZ	CCAGTCAGTCCAGGGAAGG	GTAGTCAAAGGCAGCCGTCAG	58.4	GQ201421
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	60.4	JX657682
TNF-α	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC	58.4	HQ696609
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	57.1	JQ692172
IL-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	62.3	KC535507
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	60.3	JN663841
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	61.4	HQ388294
IL-11	GGTTCAAGTCTCTTCCAGCGAT	TGCGTGTTATTTTGTTCAGCCA	57.0	KT445870
IL-12p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55.4	KF944667
IL-12p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	59.0	KF944668
IL-15	CCTTCCAACAATCTCGCTTC	AACACATCTTCCAGTTCTCCTT	61.4	KT445872
IL-17D	GTGTCCAGGAGAGCACCAAG	GCGAGAGGCTGAGGAAGTTT	62.3	KF245426
IL-4/13A	CTACTGCTCGCTTTCGCTGT	CCCAGTTTTCAGTTCTCTCAGG	55.9	KT445871
IL-4/13B	TGTGAACCAGACCCTACATAACC	TTCAGGACCTTTGCTGCTTG	55.9	KT625600
TGF-β1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	55.9	EU099588
TGF-β2	TACATTGACAGCAAGGTGGTG	TCTTGTTGGGGATGATGTAGTT	55.9	KM279716
NF-κB p52	TCAGTGTAACGACAACGGGAT	TACTTCAGCCACACCTCTCTTAG	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
GAPDH	GTTACAAGGGAGAAGTTCACCAT CCGGTA GACTCG ACTACA TACAG	61.4	GQ266395	

* LEAP-2, liver expressed antimicrobial peptide 2; IgZ, immunoglobulin Z; IFN-γ2, interferon γ2; TNF-α, tumor necrosis factor α; IL, interleukin; TGF-β, transforming growth factor β; NF-κB, nuclear factor kappa B; IκBα, inhibitor of κBα; IKK, IκB kinase; TOR, target of rapamycin; S6K1, ribosomal protein S6 kinases 1; 4E-BP, eIF4E-binding proteins; GAPDH, glycer-aldehyde-3-phosphate dehydrogenase.

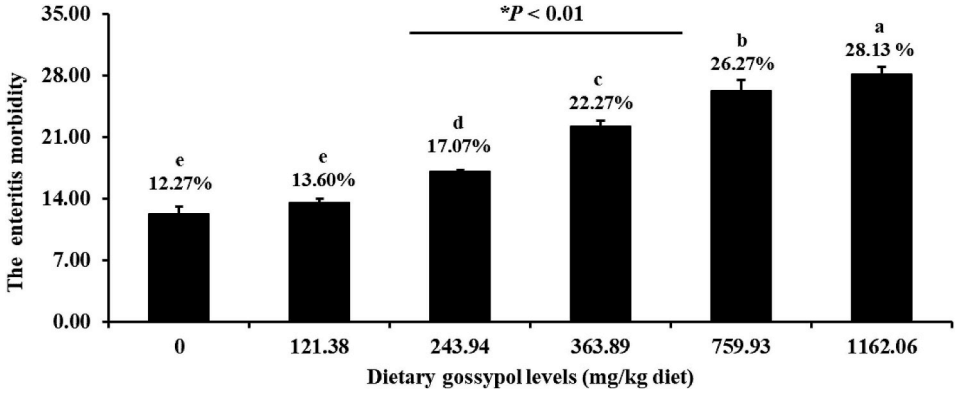

Fig. 1. Effects of dietary gossypol (mg/kg diet) on enteritis morbidity of on-growing grass carp after infection of *A. hydrophila*. Values having different letters are significantly different ($P < 0.05$). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

2.2. Challenge trial and sample collection

After a 60-days feeding trial, a challenge trial was conducted to investigate the influence of dietary gossypol 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. [34]. Aeromonas *hydrophila* (FDL20120711) was kindly provided by the College of Veterinary Medicine, Sichuan Agricultural University, China. Fifteen fish from each treatment group were randomly collected with similar body weights and moved to labeled cages, and fish were acclimatized to the

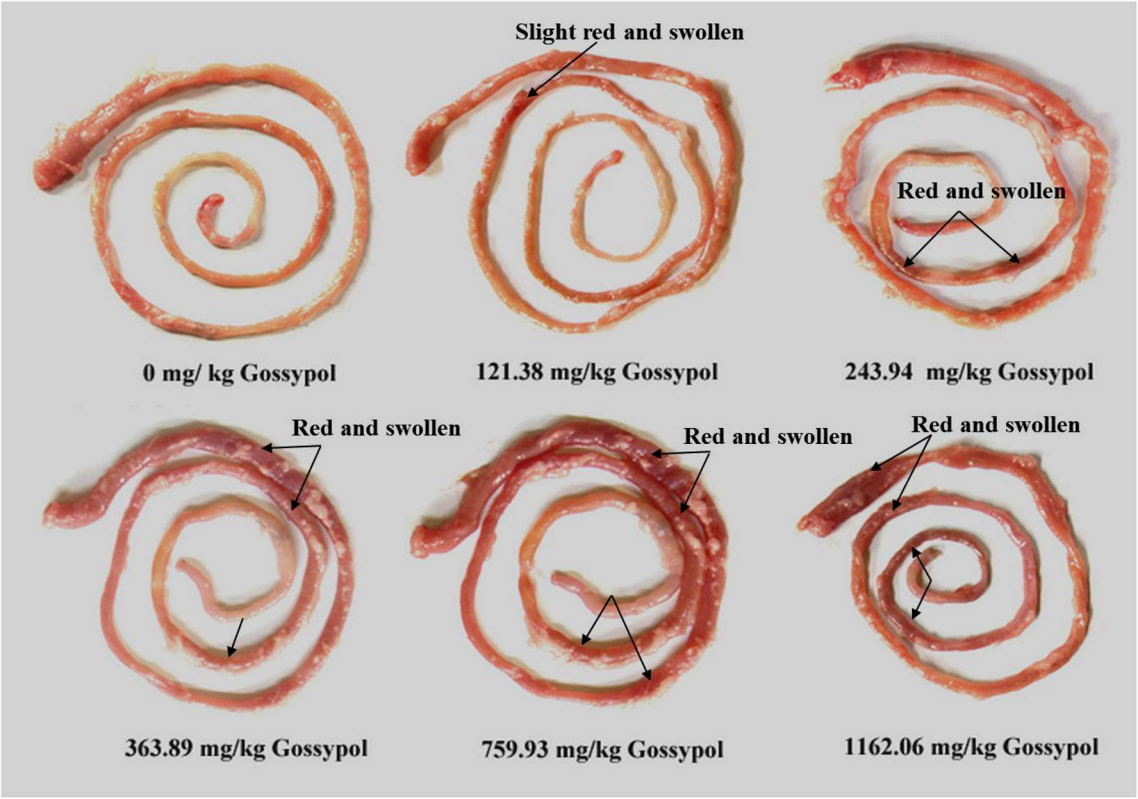

Fig. 2. Enteritis symptom of on-growing grass carp (*Ctenopharyngodon idella*) fed diets contain different levels of gossypol after challenged with *A. hydrophila* for 14 days.

experimental condition for 5 days according to our laboratory study [35]. After 5 days acclimation, each fish was intraperitoneally injected with 1.0 ml A. *hydrophila* (2.5×10^8 colony-forming units (cfu)/ml), which was determined with a nonlethal dosage that could induce inflammation efficiently according to our preliminary test (data not shown). The challenge trial was conducted for 14 days according to Basha et al. [36] and our preliminary test.

At the end of the challenge trial, all fish were anaesthetized in a benzocaine bath as described by Guo et al. [12] then sacrificed. The fish intestines were quickly removed, segmented (proximal intestine (PI), mid intestine (MI) and distal intestine (DI)) and the severity of intestinal inflammation of fish was evaluated based on the method of Song et al. [3]; the intestines were then frozen in liquid nitrogen and stored at −80 °C until further analysis, as described by Hu et al. [37].

2.3. Histological analysis

For histological evaluation, fish were dissected to remove the intestine tissues and fixed with 4% paraformaldehyde [38]. Tissues were embedded in paraffin wax after dehydrated in a graded ethanol series and equilibrated in xylene according to standard histological techniques [39]. The tissues were further dissected into 5-μm sections according to Taylor et al. [40]. Sections were stained using standard haematoxylin and eosin (H & E) and examined by a Nikon TS100 light microscope (Nikon Corporation, Tokyo, Japan). To assess the effect of different treatments on PI, MI and DI morphology, 10 images were randomly selected from each fish in each treatment. The impacts of treatments were monitored in terms of blood capillary hyperaemia, nuclear migration, goblet cell hyperplasia, loose arrangement of enterocyte, and epithelial sloughing. The morphological changes were scored as follows: 0 = not observed, 1 = low (1–3 out of 10 images), 2 = moderate (4–6 out of 10 images), and 3 = high (7 or more out of 10 images) [41].

2.4. Biochemical parameter analysis

The intestinal samples were homogenised on ice in 10 vol (w/v) of ice-cold physiological saline and centrifuged at 6000 g for 20 min at 4 °C, and then the collected supernatants were stored for the subsequent analysis of related parameters, as described by our laboratory previous study [42]. The activity of LZ and ACP in fish intestines were determined according to the method of Zheng et al. [43]. The contents of C3, C4 and IgM were measured by using the immunoturbidimetry kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the method of Liu et al. [44] and Li et al. [45].

2.5. Quantitative real-time PCR

In this study, the quantitative real-time PCR (*q*RT-PCR) was similar to the previously described in our previous study [46]. The total RNAs were extracted from the PI, MI and DI using RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions. The RNA quality and quantity were assessed by agarose gel (1%) electrophoresis and spectrophotometric analysis (A260:280 nm ratio). Subsequently, RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa) according to the manufacturer's instructions. The *q*RT-PCR reactions were performed in triplicate on a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR® Green I Supermix (Takara, Dalian, China) based on the manufacturer's protocol. For *q*RT-PCR, specific primers were designed according to the sequences cloned in our laboratory and the published sequences of grass carp (Table 2). According to the results of our preliminary

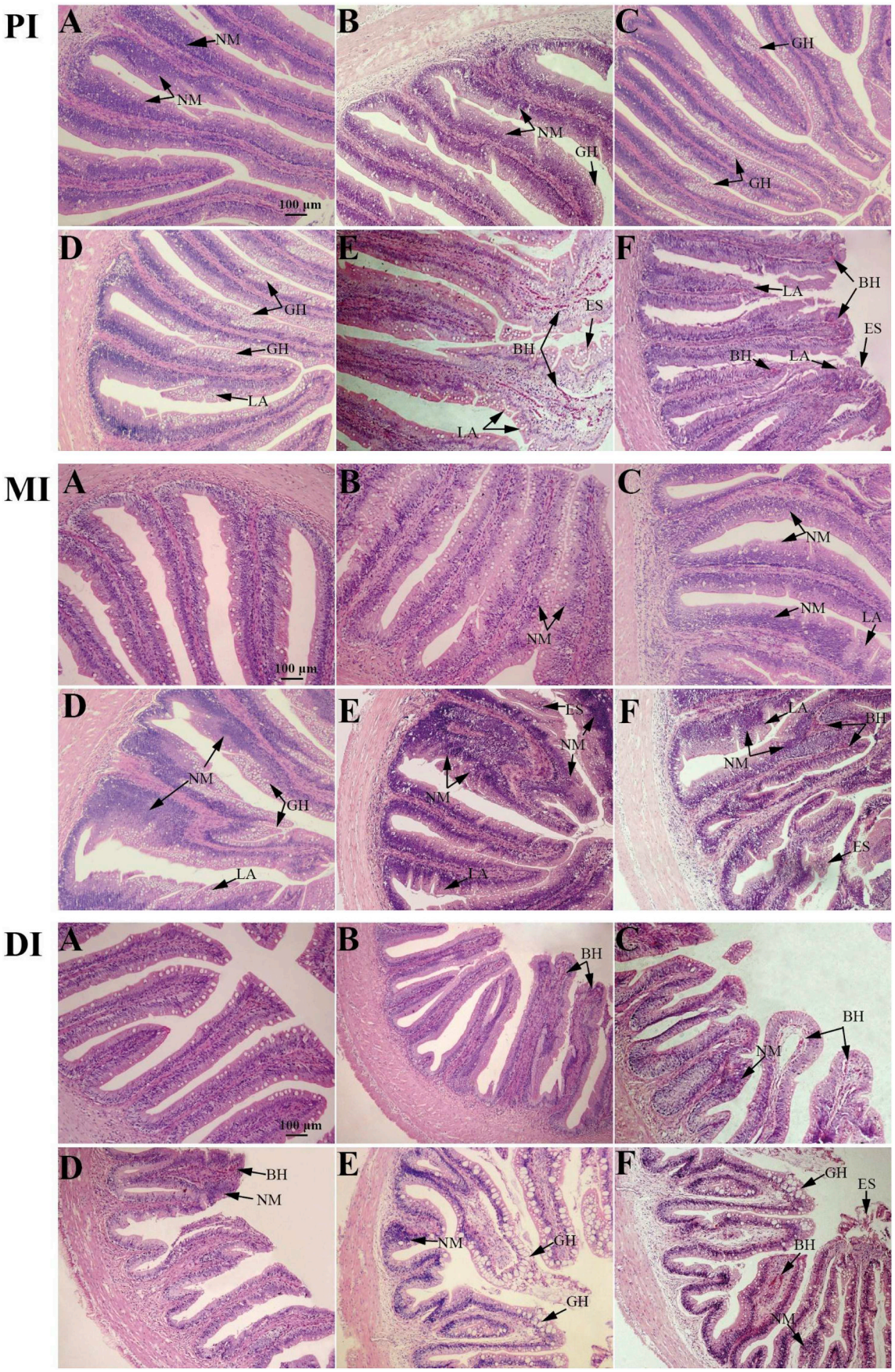

Fig. 3. The histology of the PI, MI and DI (H&E × 100) in on-growing grass carp fed diets containing different levels of gossypol (mg/kg diet) after challenged with *A. hydrophila* for 14 days. (A) Control (0), (B) 121.38 mg/kg diet, (C) 243.94 mg/kg diet, (D) 363.89 mg/kg diet, (E) 759.93 mg/kg diet, (F) 1162.06 mg/kg diet. In each panel, NM: nuclear migration, GH: goblet cell hyperplasia, BH: blood capillary hyperaemia, LA: loose arrangement of enterocyte, ES: epithelial sloughing.

Table 3
Intestinal morphological changes in different groups of on-growing grass carp fed diets containing different levels of gossypol for 60 days after infection with the *A. hydrophila* for 14 days *.

Morphology	Dietary gossypol levels (mg/kg diet)					
	Control (0)	125	250	375	775	1175
PI						
Blood capillary hyperaemia	0.67	0.67	0.67	1.00	1.33	1.67
Nuclear migration	0.67	0	0.67	1.00	1.00	1.00
Goblet cell hyperplasia	0	1.00	1.33	1.33	1.00	1.33
Loose arrangement of enterocyte	0	0	0	0.67	1.00	2.00
Epithelial sloughing	0	0	0	0	1.00	1.00
Column totals	1.34^{d}	1.67^{d}	2.67^{cd}	4.00^{bc}	5.33^{b}	7.00^{a}
MI						
Blood capillary hyperaemia	0.33	0.67	0.67	0.67	1.00	1.67
Nuclear migration	0	1.00	1.00	1.67	1.67	2.00
Goblet cell hyperplasia	1.00	0.33	0.67	1.00	1.00	1.33
Loose arrangement of enterocyte	0	0	1.00	0.33	1.00	1.00
Epithelial sloughing	0	0	0	0	1.67	1.67
Column totals	1.33^{c}	2.00^{bc}	3.34^{bc}	3.67^{b}	6.34^{a}	7.67^{a}
DI						
Blood capillary hyperaemia	0	1.00	1.00	1.67	1.00	1.67
Nuclear migration	0	0	0.67	1.00	1.33	1.00
Goblet cell hyperplasia	0.33	0	0	0.67	1.67	1.67
Loose arrangement of enterocyte	0	0	0	0	0.33	1.00
Epithelial sloughing	0	0	0	0	0	1.00
Column totals	0.33^{c}	1.00^{c}	1.67^{c}	3.34^{b}	4.33^{b}	6.34^{a}

*The morphological changes are based on light 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 or more out of 10 images); Means, N = 3 fish. Different superscript letter means significant difference when compared to control (0).

experiment concerning the evaluation of internal control genes (data not shown), β-Actin and GAPDH were used as reference genes to normalize cDNA loading as described by Vandesompele et al. [47] and Zheng et al. [48]. The target and housekeeping gene amplification efficiency were calculated according to the specific gene standard curves generated from 10-fold serial dilutions. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression results as described by Fontagne-Dicharry et al. [49] and Chu et al. [50].

2.6. Western blot analysis

The processes for intestines protein extract preparation, antibodies and western blotting are the same as those described in our previous studies [51]. Briefly, after extraction the PI, MI and DI protein concentrations were determined, using the bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology Inc., Jiangsu, China). Protein samples were separated by SDS-PAGE and transferred to 0.45 μm PVDF membrane for western analysis. The membrane was blocked for 1h at room temperature (RT) and then incubated with primary antibody overnight at 4 °C. We used the same anti-total TOR (AF6308, 1:1000 dilution), phosphorylation of TOR on residue Ser 2448 (p-TOR Ser ^{2448}) (AF3308, 1:1000 dilution), NF-κBp65 (AF5006, 1:750 dilution), Lamin B1 (AF5161, 1:1000 dilution) and β-Actin (AF7018, 1:3000 dilution) antibodies as those in our previous studies [48] and were purchased from Affinity BioReagents (Golden, Colorado, USA). The anti-total PKA (PKA-cat) (AF7746, 1:800 dilution) and phosphorylation of PKA on threonine residue 197 (*p*-PKA-cat (Thr197)) (AF7246, 1:800 dilution) antibodies selection were according to the method of Hu et al. [51]. Briefly, alignment of amino acid sequences of peptides was used to produce polyclonal antibodies with the corresponding sequences in fish to check the specificity of the antibodies; then, preliminary experiments were performed with murine samples as a control to comparative western blots of grass carp and murine samples. Thus, the anti-PKA-cat and *p*-PKA-cat (Thr197) were also purchased from Affinity BioReagents (Golden, Colorado, USA), which were checked and successfully cross reacted with the grass carp proteins of the intestine. The sources of all above antibodies were from human, mouse and rabbit. The western bands were quantified using the NIH Image 1.63 software (National Institutes of Mental Health, Bethesda, USA).

2.7. Statistical analysis

The effects of gossypol were assessed by ANOVA followed by a linear contrast to test the dose effect of gossypol on intestinal immune parameters among treatments were assessed using the general linear models (GLM) procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Results were presented as mean ± standard error (SE). All data were

Table 4
Effects of different levels of gossypol on immune related parameters in the PI, MI and DI of on-growing grass carp (*Ctenopharyngodon idella*) *.

	Dietary gossypol levels (mg/kg diet)						SEM	*P*-value	
	0 (control)	121.38	243.94	363.89	759.93	1162.06		Linear	Quadratic
PI									
LZ	109.07^{a}	113.38^{a}	96.10^{b}	91.27^{bc}	81.12^{cd}	76.33^{d}	2.67	< 0.001	0.03
ACP	101.42^{a}	95.24^{a}	83.64^{b}	76.69^{b}	69.37^{c}	62.26^{c}	2.50	< 0.001	< 0.001
C3	18.75^{a}	18.83^{a}	18.13^{b}	17.31^{c}	16.47^{d}	16.04^{d}	0.30	< 0.001	0.40
C4	7.04^{a}	7.11^{a}	6.41^{b}	6.27^{bc}	5.84^{cd}	5.77^{d}	0.11	< 0.001	< 0.001
IgM	45.38^{a}	46.91^{a}	41.25^{b}	37.84	27.85^{c}	25.10^{c}	1.49	< 0.001	0.05
MI									
LZ	113.36^{a}	107.12^{a}	95.41^{b}	89.36^{bc}	79.44^{cd}	70.62^{d}	2.92	< 0.001	0.03
ACP	136.87^{a}	132.41^{ab}	121.36^{bc}	117.07^{cd}	107.23^{d}	106.84^{d}	2.52	< 0.001	0.02
C3	18.04^{a}	18.12^{a}	17.14^{ab}	16.41^{b}	15.04^{c}	14.85^{c}	0.26	< 0.001	0.03
C4	6.58^{a}	6.37^{a}	5.36^{b}	5.33^{bc}	4.70^{c}	4.74^{c}	0.14	< 0.001	< 0.001
IgM	68.94^{a}	65.82^{a}	58.65^{b}	50.19^{c}	49.21^{c}	42.27^{d}	1.76	< 0.001	< 0.01
DI									
LZ	83.73^{a}	81.60^{ab}	70.10^{bc}	71.14^{c}	67.28^{c}	56.51^{d}	2.06	< 0.001	0.46
ACP	107.69^{a}	104.99^{ab}	97.66^{bc}	90.67^{c}	80.84^{d}	76.39^{d}	2.21	< 0.001	0.02
C3	13.20^{a}	13.16^{a}	13.02^{a}	12.68^{a}	11.88^{b}	11.50^{b}	0.15	< 0.001	0.82
C4	5.92^{a}	5.81^{a}	5.84^{a}	5.24^{b}	4.35^{c}	4.15^{b}	0.13	< 0.001	0.15
IgM	67.80^{a}	62.14^{ab}	58.13^{bc}	53.62^{cd}	48.26^{de}	44.91^{e}	1.56	< 0.001	0.01

*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).

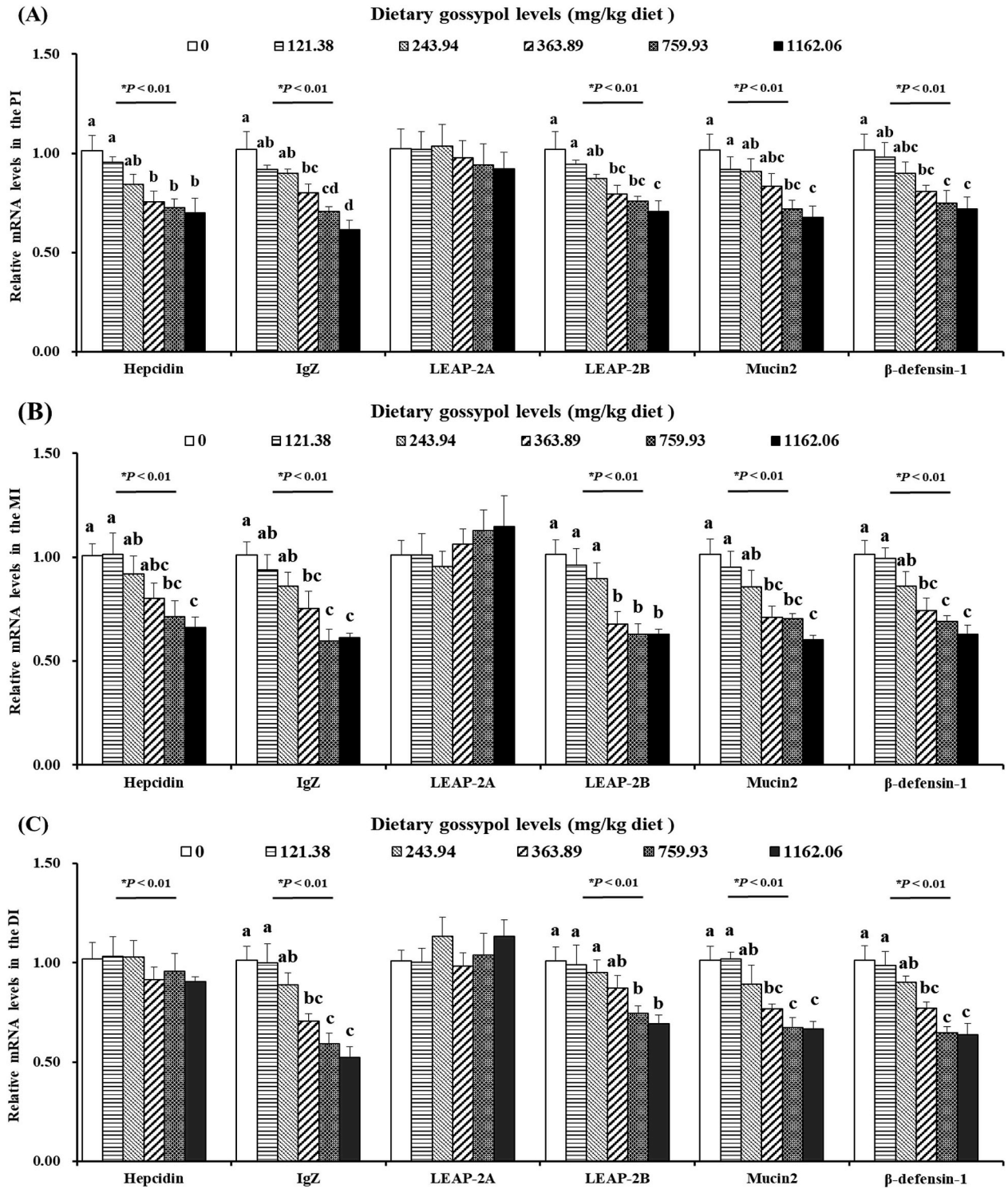

Fig. 4. Relative expression of Hepcidin, IgZ, LEAP-2A, LEAP-2B, Mucin2 and β-defensin-1 in the PI (A), MI (B) and DI (C) of on-growing grass carp fed diets containing different levels of gossypol for 60 days. Values are means (six fish per group) and standard errors represented by vertical bars. Mean values with unlike letters were significantly different ($P < 0.05$; ANOVA and Duncan's multiple-range tests). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

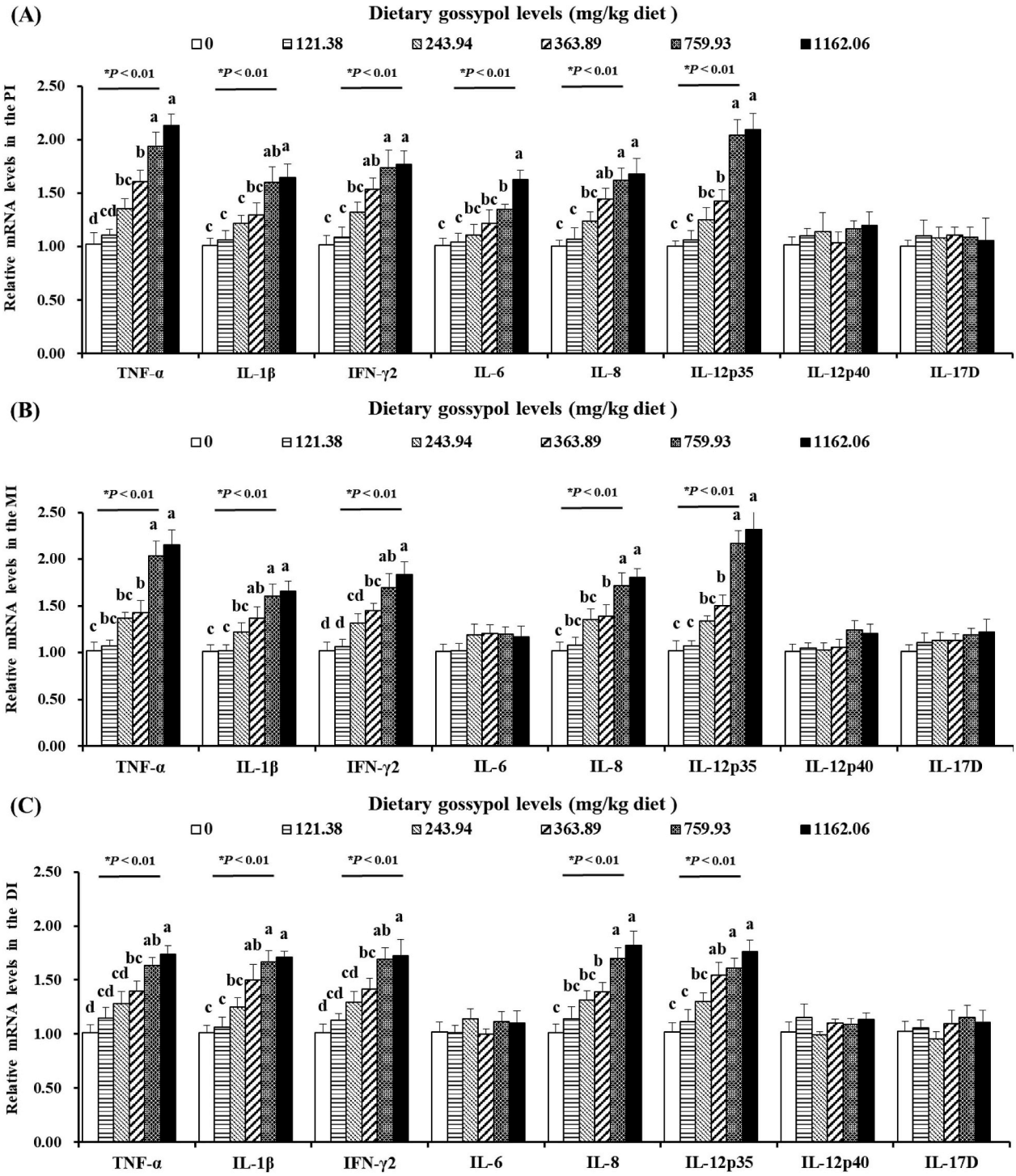

Fig. 5. Relative expression of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ2, IL-6, IL-8, IL-12p35, IL-12p40, and IL-17D) in the proximal intestine (PI) (A), middle intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp fed diets containing different levels of gossypol for 60 days. Values are means (six fish per group), and standard errors represented by vertical bars. Mean values with unlike letters were significantly different ($P < 0.05$; ANOVA and Duncan's multiple-range tests). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

subjected to one-way analysis of variance (ANOVA) followed by Duncan's method to determine significant differences among groups at the level of $P < 0.05$. The abscissa is the logarithmic value of the level of variation (Log dietary gossypol levels), and the ordinate is the statistical value of the relevant parameter, which was used for regression analysis according to Wen et al. [52]. Broken line analysis [53] was used to evaluate the safe dose of gossypol in the diets of on-growing grass carp.

3. Results

3.1. Enteritis and enteritis morbidity of fish

The enteritis severity of fish from each treatment was evaluated as described by Song et al. [54]. The results are shown in Fig. 1. Compared with the control group, increasing the levels of dietary gossypol linearly aggravated ($P < 0.05$) the enteritis morbidity of fish under *A.*

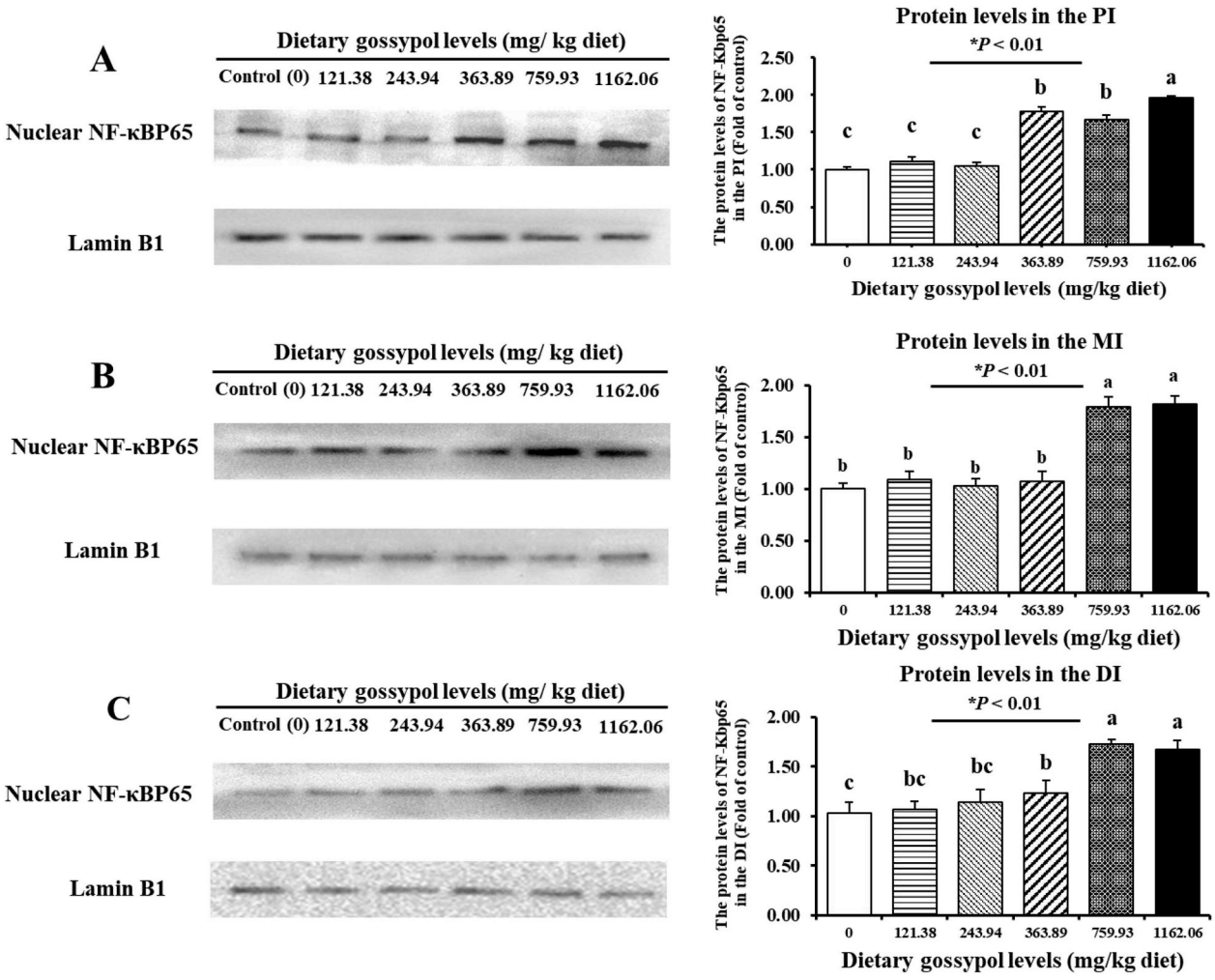

Fig. 6. Western blot analysis of NF-κBP65 in the proximal intestine (PI) (A), middle intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp fed diets containing different levels of gossypol for 60 days. Values are means (three replicates per group), and standard errors represented by vertical bars. Mean values with unlike letters were significantly different between treatments ($P < 0.05$; ANOVA and Duncan's multiple-range tests). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

hydrophila infection.

As shown in Fig. 2. The intestinal hyperemia symptom became more severe with increasing dietary gossypol levels from 121.38 to 1162.06 mg/kg diet. In addition, the survival rate of all group after infection with *A. hydrophila* was 100% (unpublished data).

3.2. Histopathological examination

The histological results are shown in Fig. 3. Compared with the control group, increasing the dietary levels of gossypol resulted in blood capillary hyperaemia, nuclear migration, goblet cell hyperplasia, loose arrangement of enterocyte, and epithelial sloughing in three intestinal segments of on-growing grass carp. A summary of the morphological changes observed in the treatments is presented in Table 3. A general trend was noticed; compared with the control group, dietary gossypol could aggravate intestinal injuries in on-growing grass carp after infection with *A. hydrophila.* Among the groups challenged with *A. hydrophila*, the histological changes in the PI, MI and DI of fish were increased significantly with the dietary gossypol level up to 363.89, 243.94 and 363.89 mg/kg diet ($P < 0.05$), respectively.

3.3. Immune parameters in the intestines of fish

The activities of LZ and ACP and the contents of C3, C4 and IGM in the three intestinal segments of on-growing grass carp after infection with *A. hydrophila* are presented in Table 4. In the PI, MI and DI of fish, the contents of C3 and C4 were linearly and quadratically (except C3 in the PI and DI) decreased ($P < 0.05$) by increasing dietary gossypol concentrations. Meanwhile, feeding diets containing different levels of gossypol linearly and quadratically decreased ($P < 0.05$) the activities of the LZ, ACP and IgM in the three intestinal segments of fish.

3.4. Relative mRNA levels of innate and adaptive components and cytokines in fish intestines

The effect of dietary gossypol on antimicrobial peptides in the PI, MI and DI of on-growing grass carp are presented in Fig. 4. The mRNA levels of Hepcidin (except DI), immunoglobulin Z (IgZ), LEAP-2B, Mucin2 and β-defensin-1 were linearly down-regulated ($P < 0.05$) in the three intestinal segments of fish with increasing dietary gossypol levels. However, the dietary gossypol did not impact on the mRNA

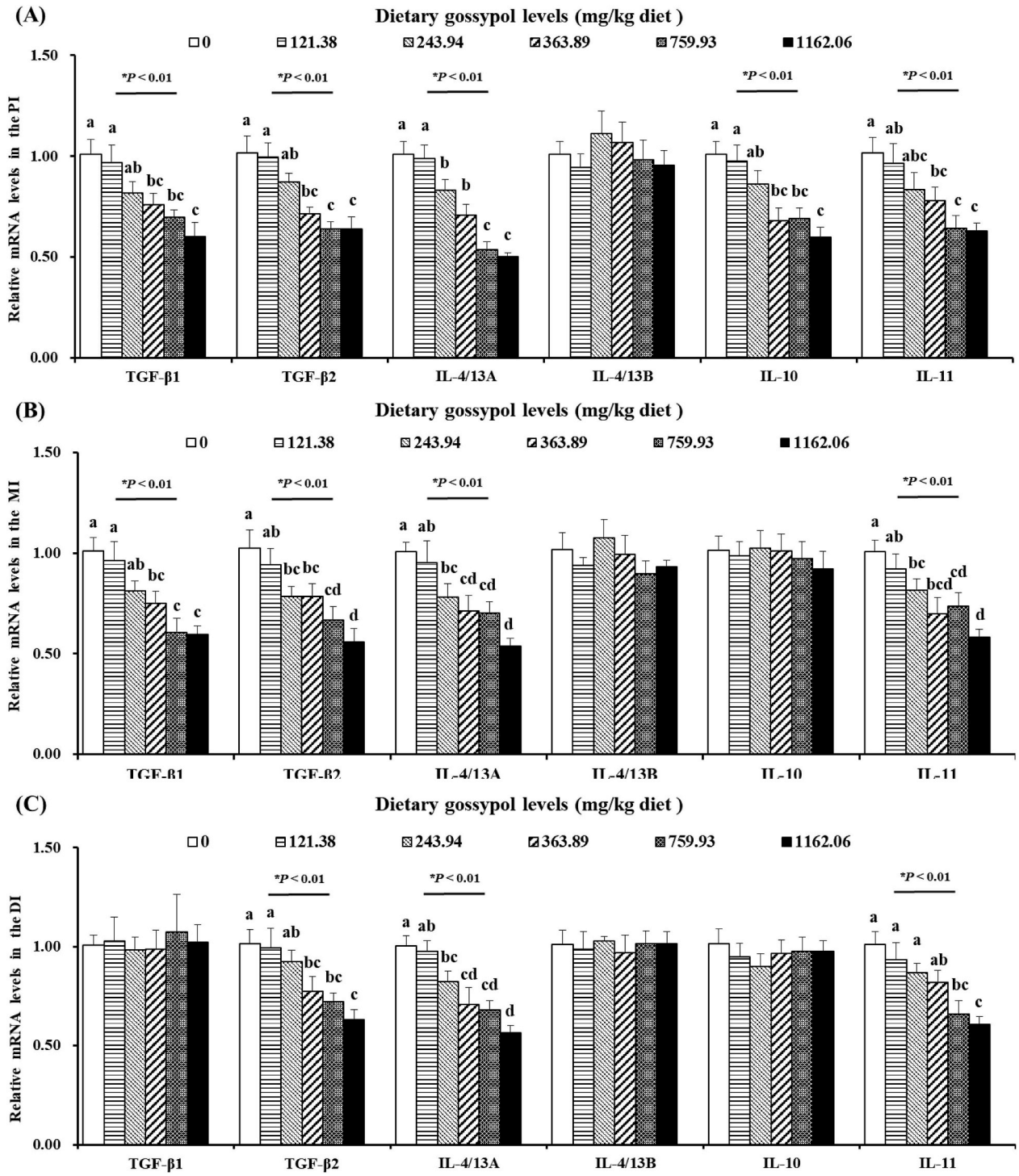

Fig. 7. Relative expression of anti-inflammatory cytokines (TGF-β1, TGF-β2, IL-4/13A, IL-4/13B, IL-10 and IL-11) in the PI (A), MI (B) and DI (C) of on-growing grass carp fed diets containing different levels of gossypol for 60 days. Values are means (six fish per group), and standard errors represented by vertical bars. Mean values with unlike letters were significantly different ($P < 0.05$; ANOVA and Duncan's multiple-range tests). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

levels of LEAP-2A in the three intestinal segments ($P > 0.05$), and Hepcidin in the DI ($P > 0.05$) of on-growing grass carp.

Effects of dietary gossypol on pro-inflammatory cytokines in the intestines of on-growing grass carp are shown in Fig. 5. The mRNA levels of TNF-α, IL-1β, IFN-γ2, IL-8, IL-12P35 and IL-6 (only in PI) were linearly down-regulated ($P < 0.05$) in the three intestinal segments of fish with increasing dietary gossypol levels. However, the dietary gossypol had no impact on the mRNA levels of IL-12P40, IL-17D and IL-6 (except PI) in the intestines of on-growing grass carp ($P > 0.05$).

The effects of dietary gossypol on anti-inflammatory cytokines genes expression in the intestines of on-growing grass carp are present in Fig. 7. The TGF-β2, IL-4/13A and IL-11 mRNA levels were linearly down-regulated ($P < 0.05$) in the three intestinal segments of fish with increasing dietary gossypol levels. The mRNA levels of TGF-β1 in the PI and MI, and IL-10 in the PI of fish were linearly down-regulated ($P < 0.05$) by increasing dietary gossypol levels. However, compared

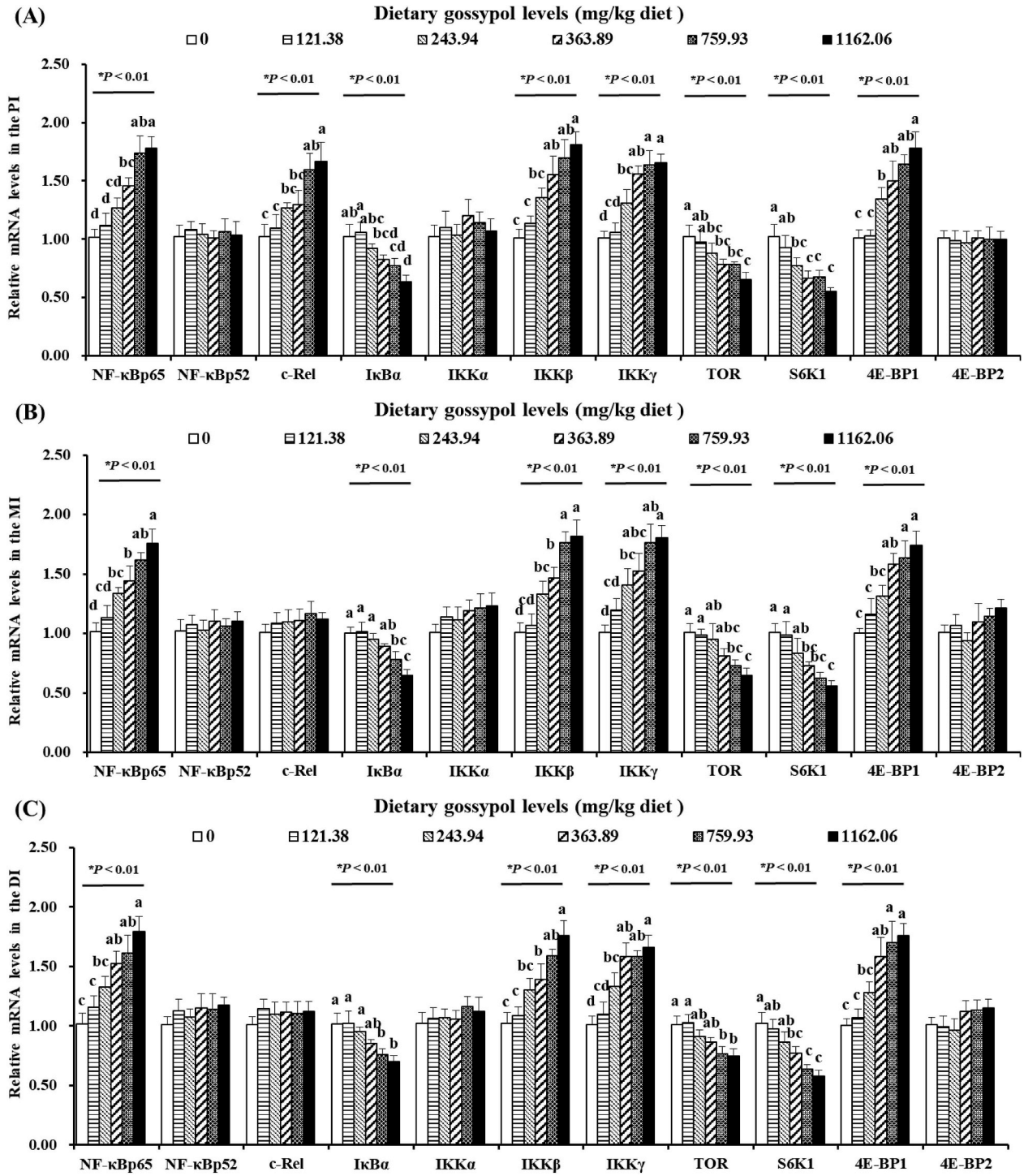

Fig. 8. Relative expression of signalling molecules in the PI (A), MI (B) and DI (C) of on-growing grass carp fed diets containing different levels of gossypol for 60 days. Values are means (six fish per group), and standard errors represented by vertical bars. Mean values with unlike letters were significantly different ($P < 0.05$; ANOVA and Duncan's multiple-range tests). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

with the control group, increasing the dietary levels of gossypol had no effect on the mRNA levels of TGF-β1 in the DI, IL-10 in the MI and DI, and IL-4/13B in the three intestinal segments of on-growing grass carp ($P > 0.05$).

3.5. Relative mRNA levels of immune-related signalling molecules in the intestines of fish

As shown in Fig. 8, compared with the control group, increasing the level of dietary gossypol linearly up-regulated ($P < 0.05$) the mRNA levels of NF-κBp65, IKKβ, IKKγ and 4E-BP1 in the three intestinal segments, and c-Rel in the MI and DI of fish. In addition, the mRNA levels of IκBα, TOR and S6K1 were linearly down-regulated ($P < 0.05$) in the three intestinal segments by increasing dietary gossypol levels. Interestingly, the mRNA levels of NF-κBp52, IKKα and 4E-BP2 in the three intestinal segments, and c-Rel in the MI and DI of fish were no differences ($P > 0.05$) occurred among all treatments.

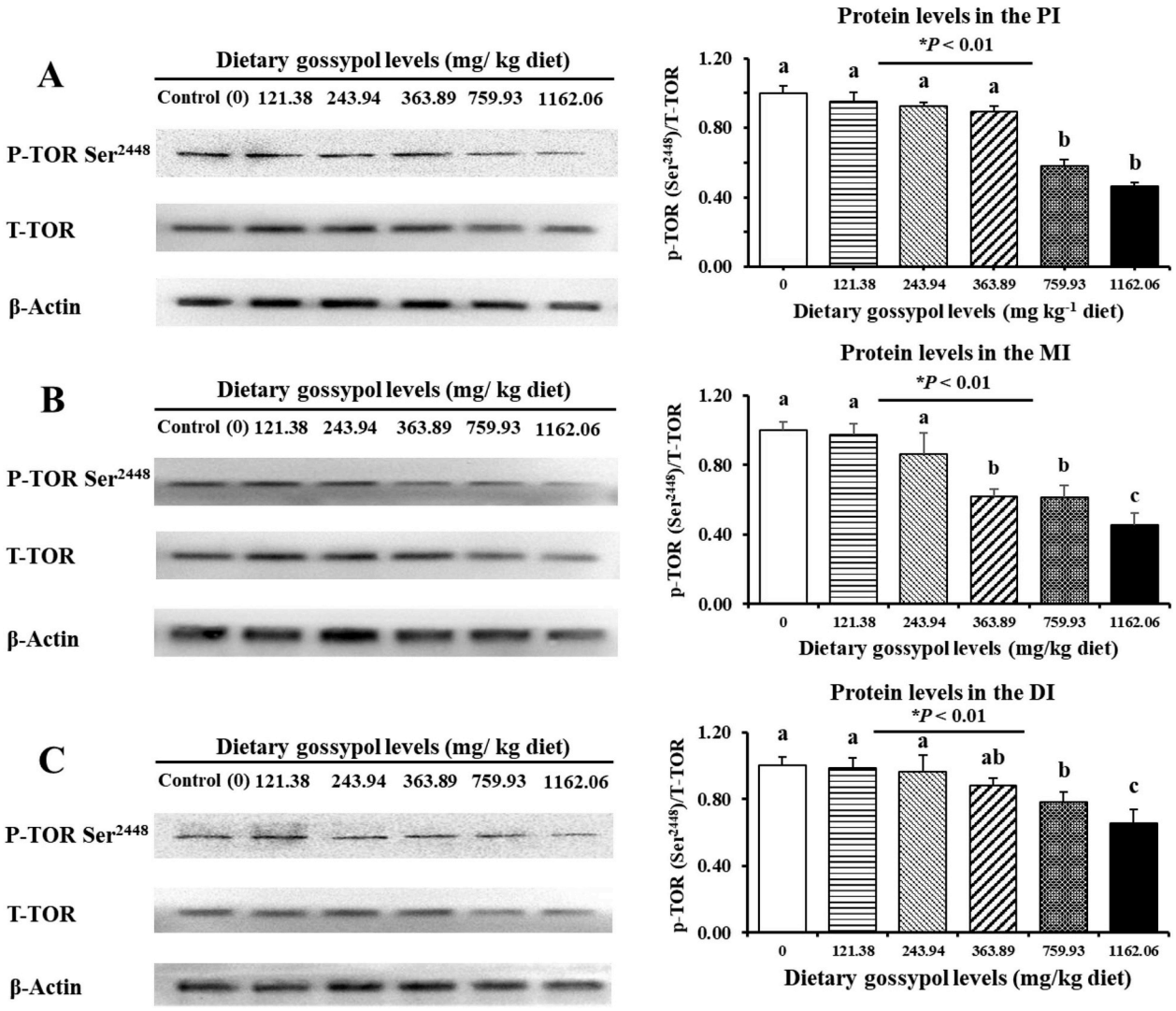

Fig. 9. Western blot analysis of total target of rapamycin (T-TOR) protein phosphorylation at Ser^{2448} (p-TOR Ser^{2448}) in the proximal intestine (PI) (A), middle intestine (MI) (B) and distal intestine (DI) (C) of fish fed diets containing different levels of gossypol for 60 days. Values are means (three replicates per group), and standard errors represented by vertical bars. Mean values with unlike letters were significantly different between treatments ($P < 0.05$; ANOVA and Duncan's multiple-range tests). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

3.6. Effects of gossypol on the protein levels of NF-κBp65, TOR and PKA in fish intestines

As shown in Fig. 6, Fig. 9 and Fig. 10, although similar quantities of proteins were loaded onto the gels, the NF-κBp65, p-TOR Ser^{2448} and *p*-PKA-cat (Thr197) protein levels were differences in the intestinal segments of fish in a dose-dependent manner. Compared with the control group, increasing the level of dietary gossypol linearly raised ($P < 0.05$) the protein levels of NF-κBp65 in the PI, MI and DI of fish. Additionally, the TOR and PKA phosphorylation levels were linearly reduced ($P < 0.05$) in the three intestinal segments of fish by increasing dietary gossypol levels.

4. Discussion

In cottonseed meal, gossypol is a toxic compound, which could reduce the growth and damage multiple organs in animals [55,56]. Our previous study observed that increasing dietary levels of gossypol above 179 mg/kg diet could decrease the growth (the SGR and PWG of Groups 1–6 were 1.87 ± 0.01^{a}, 1.85 ± 0.02^{a}, 1.80 ± 0.02^{b}, 1.69 ± 0.01^{c}, 1.60 ± 0.02^{d}, 1.54 ± 0.02^{e} and 206.88 ± 2.61^{a}, 202.76 ± 4.01^{a}, 194.89 ± 3.98^{b}, 175.81 ± 2.00^{c}, 161.51 ± 3.29^{d}, 152.60 ± 2.54^{e}, respectively) and impair the structural integrity of intestinal epithelial cells in on-growing grass carp [5]. We all know that animal growth was also related to the intestinal immunity. However, no study has been reported about the effect of gossypol on the intestinal immunity of animals. Thus, this study further investigated the effects of gossypol on the intestinal immunity and the potential regulatory mechanisms, which may afford a partial theoretical basis for the negative effect of gossypol on the growth of animals.

4.1. Gossypol could aggravate enteritis and intestinal histopathological lesions of on-growing grass carp under A. hydrophila infection

A. hydrophila is generally considered a major pathogen in almost all animal taxa and causes intestinal inflammation in fish [57]. Our previous study demonstrated that the higher enteritis morbidity could reflect the weaker enteritis resistance of fish [6]. In this study, we demonstrated for the first time that dietary gossypol was linearly increased the enteritis morbidity in on-growing grass carp, indicating that gossypol could reduce the enteritis resistance ability of fish.

Fish intestinal health was associated with its structural integrity

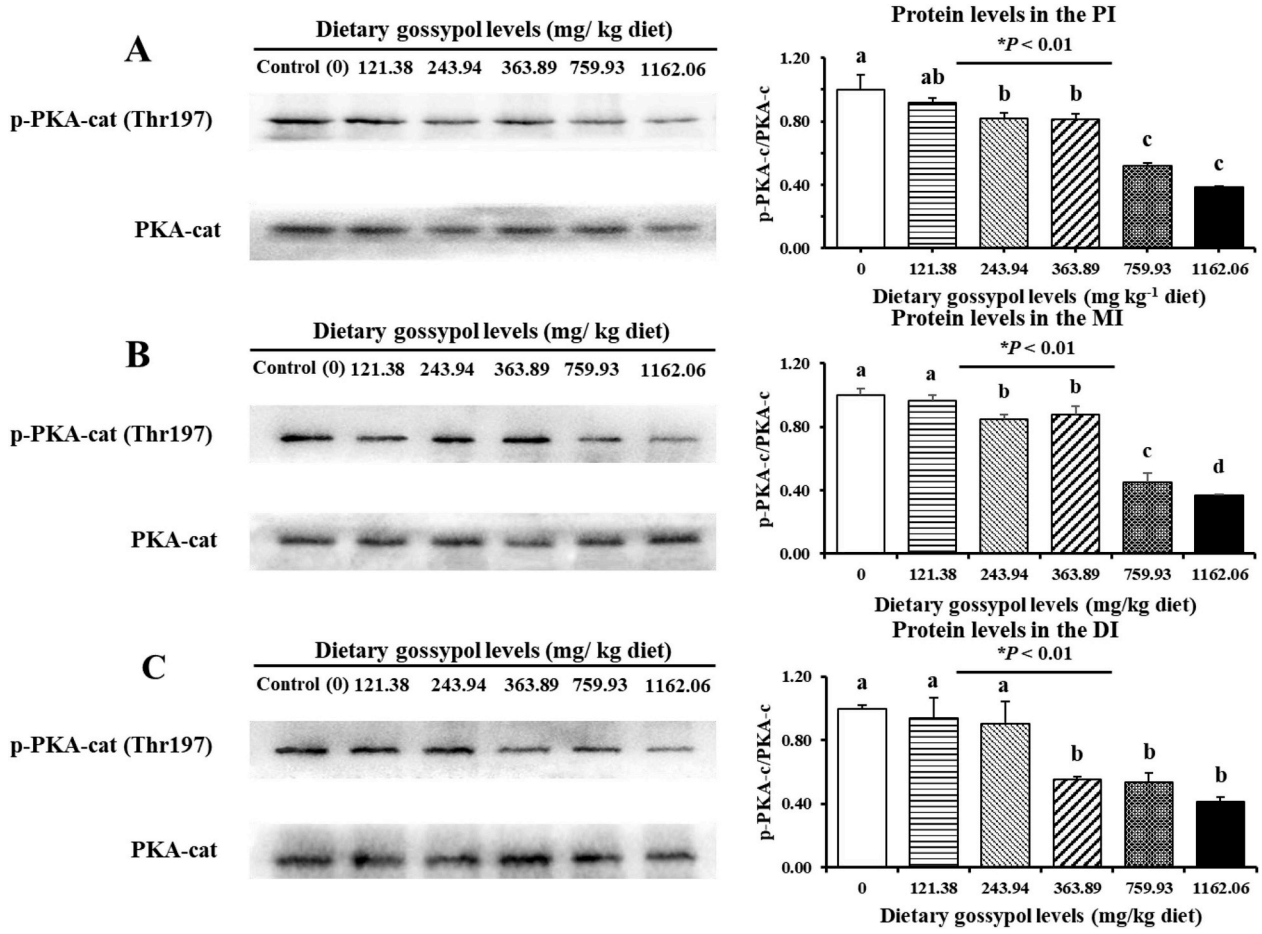

Fig. 10. Effect of gossypol on the status of PKA activation (phosphorylation) in the proximal intestine (PI) (A), middle intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp. Anti-PKA-cat immunoblot (total protein) served as internal loading control. Values are means (three replicates per group), and standard errors represented by vertical bars. Mean values with unlike letters were significantly different between treatments ($P < 0.05$; ANOVA and Duncan's multiple-range tests). *P-values underlined with a solid line indicate a significant linear dose response relationship ($P < 0.05$).

[58]. Studies have reported that blood capillary hyperaemia [59], displacement of enterocyte nuclei [60], goblet cell hyperplasia [61], loose arrangement of epithelial cells and epithelial sloughing [62] were reflected the histopathological changes in enteropathy of fish. In this study, the histopathology showed that the intestines from fish fed doses of gossypol above 121.38 mg/kg diet exhibited significant mild to severe damage.

All the data above indicated that gossypol could decrease the enteritis resistance ability and damage the intestinal structure of on-growing grass carp. Additionally, the enteritis resistance is related to intestinal immunity, which mainly depends on the innate and adaptive immune components in fish [8]. Thus, we next investigated the impacts of dietary gossypol on intestinal immunity of fish.

4.2. Gossypol reduced innate and adaptive immunity in the intestines of fish

The intestine constitutes the largest body area is continuously contacted with the external pathogens and plays a vital role in the immune defense against inflammation and pathogen infection [1]. As we know, the immune function of fish relies on the immune response, which is closely associated with innate and adaptive immune components such as LZ, ACP, complements, antibacterial peptides and immunoglobulins [63]. In the present study, dietary gossypol decreased the LZ and ACP activities, C3, C4 and IgM contents, and it down-regulated the Hepcidin (rather than DI), LEAP-2B, Mucin2 and β-defensin-1 mRNA levels in the three intestinal segments of on-growing grass carp, indicating that gossypol could impair the intestinal immune function of fish.

Interestingly, we observed that dietary gossypol down-regulated Hepcidin mRNA levels in the PI and MI (rather than DI) and had no impact on the mRNA levels of LEAP-2A in the intestines of fish. The possible reasons for the diverse results are analysed as follows. First, the dietary gossypol down-regulated Hepcidin mRNA levels in the PI and MI but did not alter it in the DI of fish, which might be due to the TGF-β1. In mice hepatic cell, the inhibition of TGF-β1 could down-regulate the mRNA levels of Hepcidin [64]. Our data show that dietary gossypol did not affect TGF-β1 mRNA levels in the DI of on-growing grass carp, which supports our hypothesis. Second, dietary gossypol had no effect on the mRNA levels of LEAP-2A in the three intestinal segments of fish, which might be partially due to the unchanged IKKα. A study has been reported that IKKα could increase IL-22 expression in mice [65]. In splenocytes of rainbow trout (*Oncorhynchus mykiss*), IL-22 could up-regulate the mRNA levels of LEAP-2A [66]. In this study, the dietary gossypol did not influence the mRNA levels of IKKα in the three intestinal segments of fish, supporting our hypothesis. In addition, the fish intestinal immune function is also related to the inflammation responses, which are primarily mediated by cytokines [67] and related signalling pathways such as NF-κB and TOR [48,68].

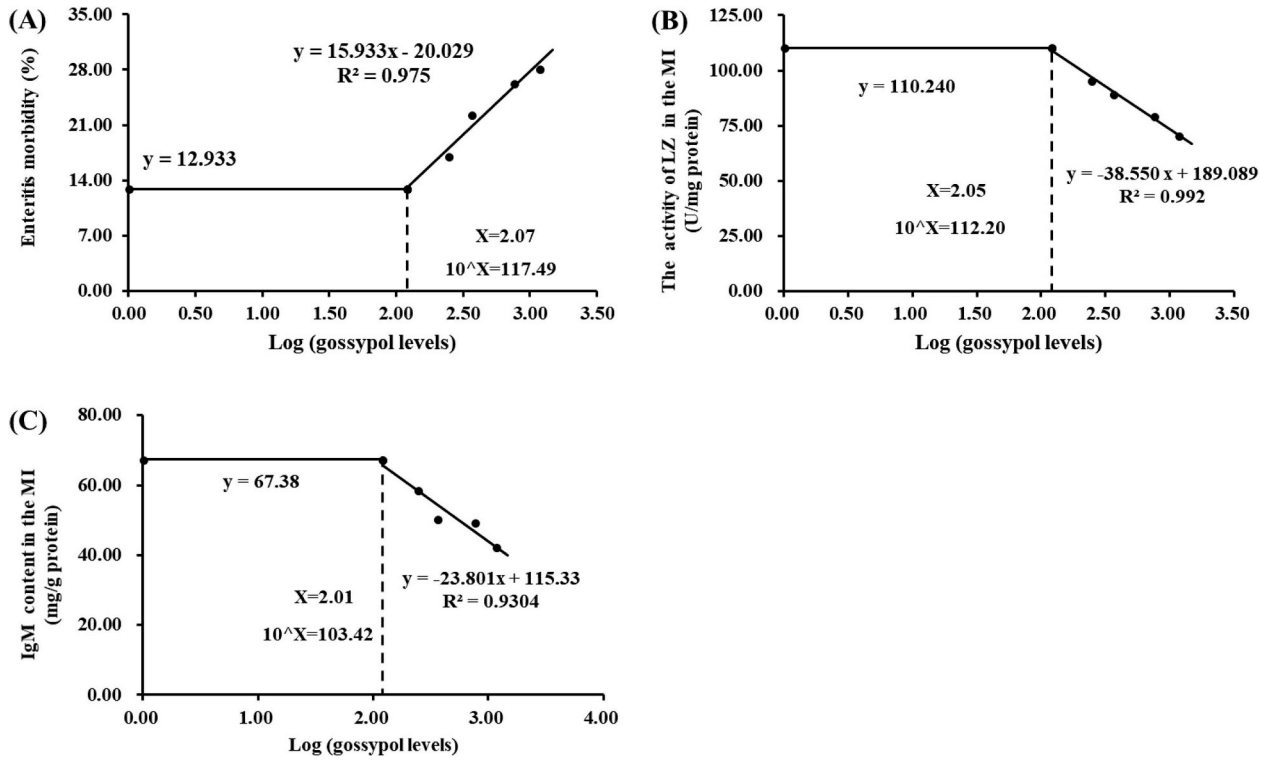

Fig. 11. Broken-line analysis of enteritis morbidity (A), LZ (B) and IgM (C) for the fish fed diets containing various levels of gossypol for 60 days. MI, middle intestine. Abscissa: the logarithm values of dietary gossypol levels.

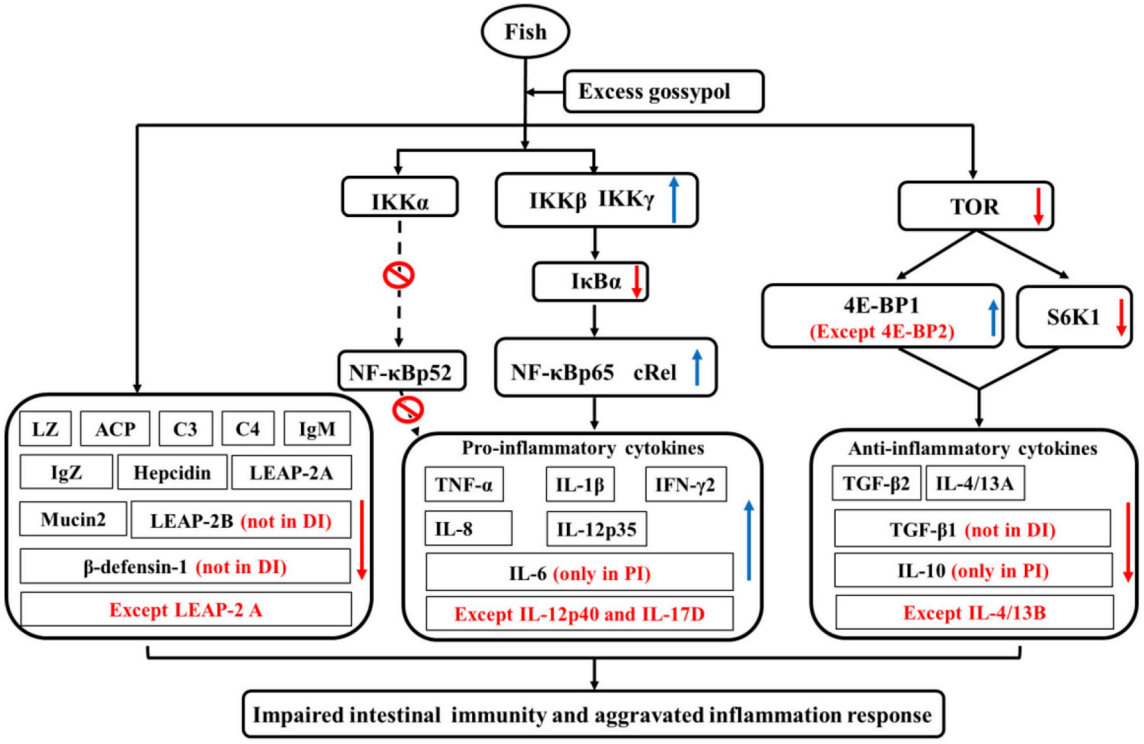

Fig. 12. The potential pathways about the effects of gossypol on immune function in the intestines of fish.

4.3. Gossypol aggravated intestinal inflammatory responses partly relating to NF-κB and TOR signalling pathways in fish

4.3.1. Gossypol increased intestinal inflammation partly related to up-regulating pro-inflammatory cytokines via the NF-κB and PKA signalling pathway in fish

In fish, enteritis induced by *A. hydrophila* infection was closely associated with the inflammatory cytokines [54]. In this study, we are the first time to evaluate the effects of gossypol on multiple pro-inflammatory cytokines mRNA levels in fish intestines. The current results indicated that dietary gossypol up-regulated the studied pro-inflammatory cytokines (except IL-6 in the MI and DI) mRNA levels in the intestines of fish, suggesting that gossypol may aggravate enteritis by up-regulating pro-inflammatory cytokines. It was reported that pro-inflammatory cytokines could be regulated by the signalling molecule NF-κB in human [69]. In addition, the inhibition of PKA could up-regulate the pro-inflammatory cytokine TNF-α in human dendritic cells [20]. Thus, we next examined the impacts of gossypol on the NF-κB and PKA signalling in different intestinal segments of on-growing grass carp.

Studies reported that activated IKK complex (IKKα, IKKβ and IKKγ) phosphorylates IκBα, results in the activation of NF-κB (e.g. NF-κBp65, p52 and c-Rel) that plays vital roles in the regulation of pro-inflammatory cytokine expression in animals [70,71]. In this study, the dietary gossypol induced the up-regulation of NF-κBp65, c-Rel (only in PI), IKK (β and γ (not *α*)) and the down-regulation of IκBα mRNA levels in the three intestinal segments of on-growing grass carp. In addition, the dietary gossypol also down-regulated the protein levels of NF-κBp65 and *p*-PKA in the three intestinal segments. These results suggest that gossypol increased the intestinal inflammation due to up-regulating the mRNA levels of pro-inflammatory cytokines, which were partially attributed to IKK (β and γ (not α))/IκBα/NF-κB (p65 and c-Rel (not p52)) and PKA signalling in the intestines of fish.

Surprisingly, no significant differences were found in the IL-6 (rather than PI), IL-12p40, IL-17D, NF-κBp52, IKKα and c-Rel (rather than PI) mRNA levels of three intestinal segments for fish fed different levels of gossypol diets. We have several explanations for this lack of effect. First, dietary gossypol up-regulated the mRNA levels of IL-6 in the PI but did not alter it in the MI and DI of fish, which might be partially associated with the c-Rel. Tumang et al. [72] reported that IL-6 was positively regulated by c-Rel in primary B cells. In this study, the dietary gossypol up-regulated c-Rel mRNA levels in the PI and did not affect it in the MI and DI of on-growing grass carp, supporting our hypothesis. Second, the dietary gossypol up-regulated IL-12p35 but did not impact on IL-12p40 mRNA levels in the three intestinal segments of fish, which might be due to IL-1β. In the mature DCs, IL-1β could up-regulate IL-12p35 mRNA level but had no effect on the mRNA level of IL-12p40 [73]. Our study showed that dietary gossypol up-regulated IL-1β mRNA levels in the three intestinal segments of fish. Thus, we speculate that gossypol might increase IL-1β gene expression leading to the up-regulation of IL-12p35 but not IL-12p40 in the intestines of fish. Third, the dietary gossypol had no impact on the mRNA levels of IL-17D in the three intestinal segments of fish, which might be due to the unchanged IKKα and NF-κBp52. It was reported that IKKα/NF-κBp52 was the non-canonical NF-κB pathway [74], which could activate the Th17 cells to produce the IL-17 family of cytokines (including IL-17A to E) [75]. Our data observed that dietary gossypol had no impact on the mRNA levels of IKKα and NF-κBp52 in the three intestinal intestines of fish. Hence, we speculated that dietary gossypol did not alter IL-17D mRNA levels in the intestines of fish might be partially associated with the unchanged mRNA levels of IKKα and NF-κBp52, which requires further investigation. Fourth, the finding that dietary gossypol did not affect NF-κBp52 mRNA levels in the three intestinal segments might be partly due to the lack of change in IKKα expression. In immune cells, IKKα plays a crucial role in the activation of NF-kBp52 [76]. In this study, the dietary gossypol did not affect IKKα mRNA levels in the three intestinal segments of fish, supporting our hypothesis. Fifth, the reason that gossypol elevated IKKβ and IKKγ but not IKKα mRNA levels in the three intestinal segments might be associated with TNF-α altering protein kinase C-zeta (PKCζ). Our study observed that dietary gossypol up-regulated TNF-α mRNA levels in the intestines of on-growing grass carp. A study in mice has been indicated that TNF-α could enhance the PKCζ levels [77], which could up-regulate IKKβ and IKKγ but not IKKα expression in kupffer cell [78]. Thus, we hypothesised that gossypol might increase PKCζ levels leading to the up-regulation of IKKβ and IKKγ (not IKKα) in the intestines of fish. However, this hypothesis requires further investigation. Lastly, gossypol up-regulated c-Rel mRNA levels in the PI but unaffected it in the MI and DI of fish, which might be related to IL-10. Rahim et al. [79] reported that the inhibition of IL-10 could up-regulate the c-Rel in macrophages. In this study, the dietary gossypol down-regulated the mRNA levels of IL-10 in the PI but unaffected it in the MI and DI of on-growing grass carp, which supports our hypothesis.

4.3.2. Gossypol induced intestinal inflammation by down-regulating the anti-inflammatory cytokines through the TOR signalling pathway in fish

In humans, the down-regulation of anti-inflammatory cytokines could aggravate the inflammation process [80], which could be regulated by TOR signalling via inhibiting ribosomal protein S6K1 and activating 4E-BP1 [81]. Our data showed that the dietary gossypol down-regulated the targeted anti-inflammatory cytokines (except TGF-β1 in the DI and IL-10 in the MI and DI) mRNA levels in the three intestinal segments of on-growing grass carp, suggesting that dietary gossypol aggravated intestinal inflammatory responses in fish. In addition, compared with the control group, the dietary gossypol down-regulated TOR and S6K1 mRNA levels, decreased TOR phosphorylation levels, and up-regulated the mRNA levels of 4E-BP1 in the three intestinal segments of fish. The above observations implied that gossypol down-regulated anti-inflammatory cytokines partially due to the suppression of the (TOR/(S6K1 and 4E-BP1)) signalling pathway in fish intestines.

Interestingly, the dietary gossypol had no effect on the IL-4/13B, IL-10 (except PI), TGF-β1 (only in DI) and 4E-BP2 mRNA levels in the three intestinal segments of fish. The possible reasons for these differences were analysed as follows. First, the dietary gossypol down-regulated IL-4/13A, but not IL-4/13B mRNA levels in the intestines of fish may be related to TOR signalling and GATA-3. The current results displayed that dietary gossypol down-regulated TOR mRNA and phosphorylation levels in the three intestinal segments of on-growing grass carp. The inhibition of TOR could down-regulate the expression of GATA3 in the T cells of mice [82]. In pufferfish, GATA3 could regulate the gene transcription of IL-4/13A but not IL-4/13B through binding with a TATA box [83]. Therefore, we specular that gossypol reduced TOR phosphorylation levels to diminish GATA-3 expression, thus leading to down-regulate the IL-4/13A (not IL-4/13B) mRNA levels in fish intestines. However, this possible supposition requires further investigation. Second, the dietary gossypol down-regulated the mRNA levels of IL-10 in the PI and had no influence it in the MI and DI of fish, which might be associated with the Cholecystokinin (CCK). A study confirmed that gossypol could bind with the lysine in feed, which in turn leads to the lysine deficiency in channel catfish [13]. Naz and Türkmen [84] found that lysine deficiency reduced the CCK secretion in larvae gilthead seabream. Meanwhile, the CCK was a higher expression in the PI than that in the MI and DI of *Schizothorax prenanti* [85]. Zhang et al. [86] observed that the inhibition of CCK could down-regulated the *IL*-10 mRNA levels in mouse liver. Hence, we speculate that dietary gossypol down-regulated the mRNA levels of IL-10 in the PI but unaffected it in the MI and DI of on-growing grass carp might be via inhibition the CCK in the intestine of fish. However, this hypothesis requires further investigation. Third, the dietary gossypol down-regulated the TGF-β1 mRNA levels in the PI and MI but did not alter it in the DI of fish, which may be associated with the intestinal isoleucine content. It was documented that isoleucine deficiency could down-regulate the mRNA levels of TGF-β1 in the intestines of grass carp [87]. Our

previous study has been found that dietary gossypol reduced the isoleucine contents in the PI and MI but did not affect it in the DI of on-growing grass carp [5], supporting our hypothesis. Lastly, the dietary gossypol up-regulated 4E-BP1 but did not alter 4E-BP2 mRNA levels in the three intestinal segments of fish, which may be related to TNF-α. A study in human L02 hepatocytes has been found that TNF-α could induce the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) [88], while the phosphorylation of eIF2α only up-regulates 4EBP1 not 4EBP2 gene expression in mouse MEF cells [89]. In this study, the dietary gossypol up-regulated TNF-α mRNA levels in the intestines of on-growing grass carp, supporting our hypothesis.

4.4. The safe dose of dietary gossypol for the intestinal immune function of fish

Our study indicated that dietary gossypol reduced the intestinal immunity of on-growing grass carp. Thus, it is necessary to evaluate the safe dose of gossypol in the diet for intestinal immunity of on-growing grass carp. As shown in Fig. 11, based on the intestinal histopathological results and the broken line analysis for the enteritis morbidity, LZ activity and IgM content in the MI, the safe dose of gossypol in the diets for on-growing grass carp were estimated to be121.38, 117.49, 112.20 and 103.42 mg/kg diet, respectively. Interestingly, on the basis of IgM, the safe dose of gossypol in the diet of on-growing grass carp was recommended to be 103.42 mg gossypol/kg diet, which was lower than that base on the percent weight gain (182.39 mg gossypol/kg diet) [5], suggesting that infections with intestinal pathogenic bacteria could decrease the gossypol tolerance of on-growing grass carp.

5. Conclusions

In summary (Fig. 12), we found for the first time that dietary gossypol impaired intestinal immune function of fish as displayed in the following aspects. Compared with the control group, (1) dietary gossypol aggravated enteritis and intestinal histopathological lesions of on-growing grass carp; (2) dietary gossypol reduced the intestinal immune function of fish by depressing the production of innate and adaptive immune components including LZ, ACP, C3, C4 and IgM and down-regulating the mRNA levels of antimicrobial peptides (Hepcidin, LEAP-2B, Mucin2 and β-defensin-1) and IgZ; (3) dietary gossypol aggravated intestinal inflammation partially by up-regulating the pro-inflammation cytokines (TNF-α, IL-1β, IFN-γ2, IL-6 (only in PI), IL-8 and IL-12p35) and down-regulating the anti-inflammatory cytokines (TGF-β1 (except DI), TGF-β2, IL-4/13A, IL-10 (only in PI) and IL-11) mRNA levels in the intestines of fish, which were partially associated with (IKK β, γ/IκBα/NF-κB (p65 and c-Rel)) and (TOR/(S6K1 and 4E-BP1)) signalling, respectively, but not (IKKα/IκBα/NF-κBp52) and (TOR/4E-BP2) signalling pathways; (4) dietary gossypol had no impact on the LEAP-2A, IL-12P40, IL-17D and IL-10 mRNA levels in the three intestinal segments of on-growing grass carp. In addition, based on the intestinal histopathological results, enteritis morbidity, LZ activity and IgM content, the safe dose of gossypol in the diets for on-growing grass carp should be less than 103.42 mg/kg diet.

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