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Deoxynivalenol decreased intestinal immune function related to NF-κB and TOR signalling in juvenile grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Deoxynivalenol (DON) is one of the most common mycotoxins in animal feed worldwide and causes significant threats to the animal production. The intestine is an important mucosal immune organ in teleost, and it is also the first target for feed-borne toxicants in animal. However, studies concerning the effect of DON on fish intestine are scarce. This study explored the effects of DON on intestinal immune function in juvenile grass carp (*Ctenopharyngodon idella*). A total of 1440 juvenile grass carp (12.17 ± 0.01 g) were fed six diets containing graded levels of DON (27, 318, 636, 922, 1243 and 1515 μg/kg diet) for 60 days. After the growth trial, fish were challenged with *Aeromonas hydrophila*. The results were analysed by the Duncan's multiple-range test ($P < 0.05$), indicating that compared with the control group (27 μg/kg diet), dietary DON levels up to 318 μg/kg diet: **(1)** decreased lysozyme (LZ) and acid phosphatase (ACP) activities, as well as complement 3 (C3), C4 and immunoglobulin M (IgM) content in the proximal intestine (PI), middle intestine (MI) and distal intestine (DI) of juvenile grass carp ($P < 0.05$); **(2)** down-regulated the mRNA levels of anti-microbial substance: liver expressed antimicrobial peptide (LEAP) -2A, LEAP-2B, hepcidin, β-defensin-1 and mucin2 in the PI, MI and DI of juvenile grass carp ($P < 0.05$); **(3)** up-regulated the mRNA levels of pro-inflammatory cytokines [interleukin 1β (IL-1β), tumour necrosis factor α (TNF-α), interferon γ2 (INF-γ2), IL-6 (only in PI), IL-8, IL-12p35, IL-12p40, IL-15 and IL-17D] in the PI, MI and DI of juvenile grass carp ($P < 0.05$), which might be partly related to nuclear factor kappa B (NF-κB) signalling [IκB kinase β (IKKβ) and IKKγ/inhibitor of κBα (IκBα)/NF-κB (p65 and c-Rel)]; and **(4)** down-regulated the mRNA levels of anti-inflammatory cytokines [IL-10, IL-11, IL-4/13A (not IL-4/13B), transforming growth factor β1 (TGF-β1) (not TGF-β2)] in the PI, MI and DI of juvenile grass carp ($P < 0.05$), which might be partly related to target of rapamycin (TOR) signalling [TOR/ribosomal protein S6 kinases 1 (S6K1) and eIF4E-binding proteins (4E-BP)]. All data indicated that DON could impair the intestinal immune function, and its potential regulation mechanisms were partly associated with NF-κB and TOR signalling pathways. Finally, based on the enteritis morbidity, and the LZ and ACP activities as well as IgM content in the PI, the reasonable dose of DON for grass carp were estimated to be 251.66, 305.83, 252.34 and 309.94 μg/kg diet, respectively.

1. Introduction

Deoxynivalenol (DON), a *Fusarium* mycotoxin, occurs widely in animal feed ingredients and feed products, and brings significant threats to the aquatic animals. Studies have observed that dietary DON caused the histopathological lesions in the caudal kidney of rainbow trout (*Oncorhynchus mykiss*) and the liver of common carp (*Cyprinus carpio* L.) [1,2], and decreased growth performance in salmonids (*Oncorhynchus mykiss*) [3]. The intestine is one of the most important mucosal immune organ in teleost [4], and it is also the first target for feed-borne toxicants in animals [5]. Intestinal health is crucial for the healthy growth of fish, which is closely related to the intestinal physical barrier and immune function [6]. However, until now, there have been only two studies referring to the intestinal immune function of fish. Our previous study showed that deoxynivalenol (DON) impaired the intestinal physical barrier of juvenile grass carp (*Ctenopharyngodon idella*) [7]. Pietsch et al. reported that DON up-regulated the mRNA levels of both pro- and anti-inflammatory cytokines in the intestine of common

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carp [8]. Therefore, a systematic and in-depth study about the effect of DON on the intestinal immune function and potential mechanisms of fish is needed.

Fish intestinal immune function is tightly correlated with innate immune components [such as lysozyme (LZ), acid phosphatase (ACP), complements and antimicrobial peptides] and adaptive immune components [such as immunoglobulins] [9]. Until now, there has been no available information about the effects of DON on innate and adaptive immune components in the intestine of fish. DON has been reported to decrease the uptake of folic acid in the intestine of mice [10]. Shi et al. revealed that low levels of folic acid reduced the LZ and ACP activities as well as complement 3 (C3) content in the intestine of grass carp [11]. In addition, DON has been reported to decrease the serum phosphorus content in pigs [12]. Chen et al. demonstrated that low levels of phosphorus resulted in the down-regulation of liver-expressed antimicrobial peptide 2 (LEAP-2) mRNA level in the intestine of grass carp [13]. Moreover, a study found that DON could increase the lipid uptake in human intestinal epithelial cells [14]. A previous study from our laboratory observed that high levels of lipids led to the decrease of immunoglobulin M (IgM) content in the intestine of grass carp [15]. These data suggest that DON might affect the intestinal immune function associated with innate and adaptive immune components of fish, which is worthy of further investigation.

Moreover, fish intestinal immune function is closely associated with cytokines, which are categorised into pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α) and interleukin (IL)-6, and anti-inflammatory cytokines, such as IL-10 and transforming growth factor β (TGF-β) [16]. Besides, studies in humans reported that the expression of pro-inflammatory cytokines could be regulated by the nuclear factor κB (NF-κB)-dependent pathway [17], whereas mammalian target of rapamycin (mTOR) signalling could regulate the expression of anti-inflammatory cytokines [18]. However, to date, there has been no report regarding the relationship between DON and cytokines as well as the related signalling pathway in the intestine of fish. One study reported that DON reduced the mRNA levels of insulin-like growth factor 1 (IGF-1) in the plasma of mice [19]. In mice, IGF-1 decreased vascular mRNA expression of TNF-α and IL-6 [20] and activated mTOR signalling in the C2C12 myoblasts [21]. Moreover, a study in pigs found that DON decreased the content of selenium in the liver [22], and low levels of selenium could enhance the nuclear translocation of NF-κBp65 in the PC3 cells of humans [23]. The above data suggest that DON might regulate the intestinal inflammatory cytokines partly related to NF-κB and TOR signalling of fish, which requires investigation.

In the present study, the growth trial was the same as our previous study, which is a part of a larger research effort to determine the effects of DON on fish growth and the intestinal physical barrier [7]. One study demonstrated that fish growth was also tightly associated with the intestinal immune function [24], so we hypothesise that DON might impair fish intestinal immune function. To confirm this assumption for the first time, we aimed to investigate the relationship between DON and innate and adaptive immune components, cytokines as well as the related signalling pathways (such as NF-κB and TOR signalling) in the intestine of grass carp, which could partially illuminate the effects of DON on intestinal immune function and its potential mechanisms in fish. Grass carp is the biggest contributor to the world's aquaculture production (FAO, 2015) [25]. In addition, a study reported that DON could be detected in more than 80% of commercial feed for cyprinids, with maximum concentrations up to 825 μg/kg [26]. Previous studies have reported that dietary DON levels up to 636 μg/kg caused body malformation and intestinal histopathological lesions in grass carp (*Ctenopharyngodon idella*) [7], and dietary DON levels up to 352 μg/kg resulted in severe hyperaemia in the liver of common carp (*Cyprinus carpio* L.) [1]. Thus, we determined the reasonable dose of DON for grass carp, which might provide a partial basis for the feed formulation of grass carp.

Table 1
Composition and nutrients content of basal diet.

Ingredients	%	Nutrients content	%
Fish meal	4.10	Crude protein[d]	32.71
Casein	27.00	Crude lipid[d]	4.55
Gelatin	7.50	n-3[e]	1.04
DL-Met (99%)	0.16	n-6[e]	0.96
L-Trp (98%)	0.03	Available phosphorus[f]	0.84
Thr (98.5%)	0.14		
Fish oil	2.62		
Soybean oil	1.81		
α-starch	23.00		
Corn starch	20.28		
Cellulose	5.00		
$Ca(H_2PO4)_2$	3.31		
Vitamin premix[a]	1.00		
Mineral premix[b]	2.00		
Ethoxyquin (30%)	0.05		
Choline chloride (50%)	1.00		
DON premix[c]	1.00		

[a] Per kilogram of vitamin premix (g/kg): retinyl acetate (500,000 IU/g), 0.39; cholecalciferol (500,000 IU/g), 0.40; D, L-α-tocopherol acetate (50%), 23.23; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.09; ascorhyl acetate (95%), 9.77; 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 corn starch to 1 kg.

[b] Per kilogram of mineral premix (g/kg): $MnSO_4 \cdot H_2O$ (31.8% Mn), 2.6590; $MgSO_4 \cdot H_2O$ (15.0% Mg), 200.0000; $FeSO_4 \cdot H_2O$ (30.0% Fe), 12.2500; $ZnSO_4 \cdot H_2O$ (34.5% Zn), 8.2460; $CuSO_4 \cdot 5H_2O$ (25.0% Cu), 0.9560; KI (76.9% I), 0.0650 g; Na_2SeO3 (44.7% Se), 0.0168. All ingredients were diluted with corn starch to 1 kg.

[c] Per kilogram of DON premix (g/kg): the DON was diluted with corn starch to 1 kg Premix was added to obtain graded levels of DON.

[d] Crude protein and crude lipid content were measured value.

[e] n-3 and n-6 content were referenced to Zeng et al. [64], and calculated according to NRC (2011).

[f] Available phosphorus content was referenced to Liang et al. [65], and calculated according to NRC (2011).

2. Material and methods

2.1. Experiments diets

As shown in Table 1, the formulation of the basal diet was the same as that described in our previous study [7]. DON (purchased from Pribolab Pte, Ltd. Singapore; purity > 98%) was added to other ingredients at six different concentrations (0 μg/kg (un-supplemented), 300 μg/kg, 600 μg/kg, 900 μg/kg, 1200 μg/kg and 1500 μg/kg final feed, respectively) according to the method of Pietsch et al. [27]. The dietary actual DON content of the six diets were determined to be 27 (un-supplemented), 318, 636, 922, 1243 and 1515 μg/kg diet, by the method of high performance liquid chromatography (HPLC) according to Pietsch et al. [27]. The diets were stored at −20 °C until feeding, which was similar to the method described by Sanden et al. [28].

2.2. Growth trial

All protocols were approved by the University of Sichuan Agricultural Animal Care Advisory Committee, Sichuan, China under permit No. HC-S20153418. The grass carp were obtained from fishers (Sichuan, China). Before initiating the experiment, all fish were accustomed to the experimental environment for four weeks as described by Su et al. [24]. Then, 1440 fish (mean weight 12.17 ± 0.03 g) were randomly distributed to 18 experimental cages (1.4 length × 1.4 width × 1.4 height in meters), resulting in 80 juvenile grass carp per cage. A disc of 100 cm in diameter was placed in the bottom of each cage to collect the uneaten feed, according to Tang et al. [29]. All of the

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

Target Gene	Primer sequence Forward (5' → 3')	Primer sequence Reverse (5' → 3')	Amplification efficiency	Temperature (°C)	Accession number
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	100.0	59.3	JQ246442.1
LEAP-2A	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	100.1	59.3	FJ390415.1
LEAP-2B	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	100.1	59.3	KT625603
Mucin2	GAGTTCCCAACCCAACACAT	AAAGGTCTACACAATCTGCCC	90.2	60.4	KT625602
β-defensin-1	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	100.0	58.4	KT445868
TGF-β1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	99.5	55.9	EU099588
TGF-β2	TACATTGACAGCAAGGTGGTG	TCTTGTTGGGGATGATGTAGTT	96.8	55.9	KM279716
IL-4/13A	CTACTGCTCGCTTTCGCTGT	CCCAGTTTTCAGTTCTCTCAGG	108.6	55.9	KT445871
IL-4/13B	TGTGAACCAGACCCTACATAACC	TTCAGGACCTTTGCTGCTTG	92.2	55.9	KT625600
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	100.2	61.4	HQ388294
IL-11	GGTTCAAGTCTCTTCCAGCGAT	TGCGTGTTATTTTGTTCAGCCA	91.1	57.0	KT445870
TNF-α	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC	99.7	58.4	HQ696609
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	99.7	60.4	FJ766439.1
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	100.4	57.1	JQ692172
IL-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	100.0	62.3	KC535507.1
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	100.2	60.3	JN663841
IL-12p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	106.8	55.4	KF944667.1
IL-12p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	95.6	59.0	KF944668.1
IL-15	CCTTCCAACAATCTCGCTTC	AACACATCTTCCAGTTCTCCTT	96.8	61.4	KT445872
IL-17D	GTGTCCAGGAGAGCACCAAG	GCGAGAGGCTGAGGAAGTTT	91.2	62.3	KF245426.1
TOR	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	100.0	61.4	JX854449
S6K1	TGGAGGAGGTAATGGACG	ACATAAAGCAGCCTGACG	100.4	54.0	EF373673
4E-BP1	GCTGGCTGAGTTTGTGGTTG	CGAGTCGTGCTAAAAAGGGTC	100.7	60.3	KT757305
4E-BP2	CACTTTATTCTCCACCACCCC	TTCATTGAGGATGTTCTTGCC	92.5	60.3	KT757306
NF-κBp65	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	99.5	62.3	KJ526214
NF-κBp52	TCAGTGTAACGACAACGGGAT	ATACTTCAGCCACACCTCTCTTAG	101.3	58.4	KM279720
c-Rel	GCGTCTATGCTTCCAGATTTACC	ACTGCCACTGTTCTTGTTCACC	92.1	59.3	KT445865
IκBα	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA	100.3	62.3	KJ125069
IKKα	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA-3	100.0	60.3	KM279718
IKKβ	GTGGCGGTGGATTATTGG	GCACGGGTTGCCAGTTTG	100.1	60.3	KP125491
IKKγ	AGAGGCTCGTCATAGTGG	CTGTGATTGGCTTGCTTT	99.9	58.4	KM079079
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	100.0	61.4	M25013–>

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

fish were fed with their respective diets to apparent satiation, four times per day. During the experimental period, the oxygen concentration was above 6.0 mg/L. The water temperature was at 28.5 ± 2.0 °C, and the pH value was at 7.5 ± 0.3. The feeding trial period was 60 days.

2.3. Challenge trial and sample collection

Aeromonas hydrophila, a widespread pathogen in freshwater, is considered a major pathogen that causes intestinal inflammation in fish, and it is usually used for establishing a model of intestinal inflammation in the grass carp [30]. Thus, in our study, the grass carp was challenged with *A. hydrophila* to investigate the effect of DON on the enteritis resistance ability and intestinal immune function of fish. The *A. hydrophila* was kindly provided by the Veterinary Medicine College of Sichuan Agricultural University in China. After the growth trial, forty-five fish from each treatment group (3 cages/group, N = 15 fish/cage) were intraperitoneally injected with 1 ml of *A. hydrophila* at a concentration of 2.5×10^6 CFU in 1 mL/fish, and then moved to six cages as described by Su et al. [24]. Meanwhile, another forty-five fish from control group (DON un-supplemented) were intraperitoneally injected with the same quantity of saline as the saline group. During the challenge trial, the experimental conditions were the same as those in the growth trial. The challenge test was conducted for 14 days according to Su et al. [24]. We selected the concentration of *A. hydrophila* and test period that could effectively induce enteritis in juvenile grass carp according to previous studies [24] and a preliminary test (data was not shown). At the end of the challenge trial, all the fish from each treatment were anaesthetised in a benzocaine bath as described by Tian et al. [31]. The degree of intestinal red colouration and swelling was selected to evaluate enteritis morbidity according to Tian et al. [31]. A scoring system was designed to evaluate the severity of fish enteritis

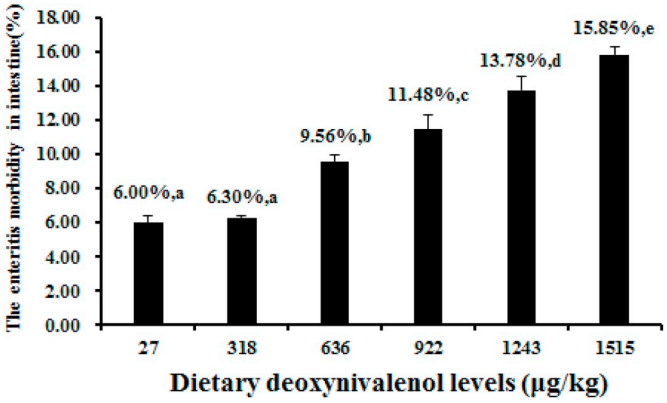

Fig. 1. Effects of graded levels of deoxynivalenol on enteritis morbidity of juvenile grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. Data represent means (n = 45 individuals in each group). Values having different letters are significantly different ($P < 0.05$).

with a semi-quantitative method according to Song et al. [30]. After sacrifice, the intestines of fish were quickly removed and segmented into proximal intestine (PI), mid intestine (MI) as well as distal intestine (DI), frozen in liquid nitrogen and stored at −80 °C for biochemical analysis, RNA extraction and western blot analysis as described by Zheng et al. [32]. Parts of the PI, MI and DI of three fish in each group were washed with physiological saline and preserved in 10% neutral buffered formalin for histological examination according to Wu et al. [33].

Table 3
Effects of graded levels of deoxynivalenol on immune related parameters in the PI, MI and DI of juvenile grass carp (*Ctenopharyngodon idella*)[a].

	Dietary DON levels (μg/kg diet)					
	27	318	636	922	1243	1515
PI						
LZ	104.64 ± 2.10^{e}	103.58 ± 3.17^{e}	89.67 ± 4.87^{d}	81.25 ± 1.83^{c}	69.42 ± 3.35^{b}	54.70 ± 5.18^{a}
ACP	192.12 ± 12.16^{c}	189.97 ± 6.36^{c}	175.10 ± 8.31^{b}	169.95 ± 16.68^{b}	158.09 ± 4.65^{a}	147.53 ± 6.86^{a}
C3	33.70 ± 1.34^{e}	32.10 ± 1.54^{e}	25.29 ± 0.74^{d}	23.42 ± 1.88^{c}	20.10 ± 1.05^{b}	15.76 ± 0.94^{a}
C4	30.89 ± 1.62^{c}	29.60 ± 0.97^{c}	26.36 ± 1.40^{b}	24.75 ± 2.09^{b}	22.62 ± 1.96^{a}	19.89 ± 0.94^{a}
IgM	111.18 ± 9.38^{d}	110.46 ± 4.52^{d}	101.58 ± 7.66^{c}	92.18 ± 8.12^{b}	81.32 ± 6.52^{a}	75.19 ± 5.03^{a}
glucose	28.62 ± 1.70^{e}	27.13 ± 2.39^{e}	24.67 ± 1.58^{d}	22.64 ± 1.11^{c}	20.35 ± 1.12^{b}	17.21 ± 1.32^{a}
MI						
LZ	148.40 ± 6.92^{e}	142.05 ± 8.01^{e}	133.84 ± 7.68^{d}	123.20 ± 5.52^{c}	111.30 ± 7.37^{b}	101.00 ± 3.40^{a}
ACP	204.64 ± 14.68^{d}	200.82 ± 7.45^{d}	177.78 ± 5.27^{c}	172.77 ± 12.92^{bc}	160.96 ± 14.41^{b}	145.80 ± 7.11^{a}
C3	39.50 ± 3.70^{d}	37.60 ± 2.18^{d}	33.80 ± 2.88^{c}	29.02 ± 2.90^{b}	26.46 ± 1.17^{ab}	24.27 ± 0.97^{a}
C4	30.30 ± 2.85^{c}	29.44 ± 0.78^{c}	27.14 ± 1.70^{b}	26.87 ± 1.90^{b}	25.40 ± 1.02^{b}	22.48 ± 2.03^{a}
IgM	100.46 ± 8.21^{d}	97.82 ± 6.90^{d}	88.23 ± 7.52^{c}	79.81 ± 5.63^{b}	74.98 ± 2.36^{ab}	68.15 ± 2.42^{a}
glucose	30.14 ± 1.90^{d}	27.10 ± 2.48^{c}	25.67 ± 1.10^{c}	21.73 ± 1.15^{b}	19.65 ± 1.45^{a}	17.95 ± 1.56^{a}
DI						
LZ	116.11 ± 2.01^{e}	115.04 ± 2.70^{e}	111.55 ± 2.86^{d}	106.77 ± 2.62^{c}	101.11 ± 3.40^{b}	97.25 ± 3.19^{a}
ACP	194.33 ± 17.19^{c}	190.64 ± 13.36^{c}	175.50 ± 11.52^{b}	168.72 ± 9.55^{b}	161.91 ± 11.79^{ab}	153.07 ± 2.46^{a}
C3	34.00 ± 1.47^{e}	32.63 ± 2.40^{e}	27.91 ± 1.77^{d}	24.79 ± 1.74^{c}	19.47 ± 0.79^{b}	17.40 ± 1.03^{a}
C4	37.97 ± 2.51^{d}	36.59 ± 2.67^{d}	30.38 ± 1.83^{c}	28.47 ± 2.77^{c}	24.98 ± 1.70^{b}	20.79 ± 1.20^{a}
IgM	82.10 ± 6.97^{b}	81.14 ± 6.43^{b}	75.49 ± 6.54^{ab}	74.06 ± 5.23^{ab}	69.90 ± 4.22^{a}	66.04 ± 2.93^{a}
glucose	33.28 ± 2.22^{e}	32.26 ± 1.97^{e}	28.60 ± 2.25^{d}	26.40 ± 2.13^{c}	21.70 ± 0.47^{b}	17.00 ± 1.00^{a}
Regression						
$Y_{LZ\ in\ PI} = -0.0393x + 116.1267$			$Y_{max} = 104.1077$		$R^2 = 0.9914$	$P < 0.01$
$Y_{ACP\ in\ PI} = -0.0340x + 199.6260$			$Y_{max} = 191.0465$		$R^2 = 0.9873$	$P < 0.01$
$Y_{IgM\ in\ PI} = -0.0303x + 120.2066$			$Y_{max} = 110.8153$		$R^2 = 0.9967$	$P < 0.01$

[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); glucose (μmol/g wet tissue).

2.4. Biochemical analysis

The intestinal samples were homogenised in 10 vol (w/v) of ice-cold physiological saline and centrifuged at 6000 ***g*** for 20 min at 4 °C, and the supernatants were used for biochemical analysis as described by our previous laboratory study [34]. The activity of LZ was measured according to the method of Liu et al. [35]. ACP activity was spectrophotometrically measured with an acid phosphatase detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to Zhou et al. [36]. The content of complement C3 and C4 in the intestine were determined based on the principle of immunoturbidimetry according to Li et al. [37]. The content of IgM was determined according to the instruction of the commercial kit, which was purchased from Zhejiang Elikan Biological Technology CO., LTD as described by Zheng et al. [38]. The glucose content was determined as described by Sergio et al. [39].

2.5. Histological examination

The intestines were fixed in 10% neutral buffered formalin, dehydrated in ethanol/methanol and embedded in paraffin according to Kokou et al. [40]. The tissue was sectioned at 4 μm. The sections were stained using standard haematoxylin and eosin (H & E) and examined by a light microscope (Nikon Eclipse TS100, Nikon Corporation, Tokyo, Japan) according to the method of Kokou et al. [40].

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

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were performed according to our previous study [31]. Total RNA was extracted from the PI, MI and DI using RNAiso Plus Kit (Takara, Dalian, China) according to the manufacturer's instructions followed by DNAse I treatment. Then, the quality and quantity were assessed using agarose gel (1%) electrophoresis (supplementary materials Fig. S4) and spectrophotometric (A260:280 nm ratio) analysis (supplementary materials Table. S1), respectively, as described by Yousefi et al. [41]. Subsequently, RNA was reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa) according to the manufacturer's instructions. For quantitative real-time PCR, specific primers were designed according to the sequences cloned in our laboratory and the published sequences of grass carp (Table 2). According to the results of our preliminary experiment concerning the evaluation of internal control genes (data not shown), β-actin was used as a reference gene to normalise cDNA loading. The target and housekeeping gene amplification efficiency were calculated according to the specific gene standard curves generated from 10-fold serial dilutions. The $2^{-\Delta\Delta CT}$ method was used for calculating the expression results after verifying that the primers amplified with an efficiency of approximately 100% according to Livak and Schmittgen [42].

2.7. Protein extraction and western blot analysis

The procedures for intestinal protein extract preparation, antibodies and western blotting were conducted according to a previous study [43]. Briefly, the protein concentrations were determined by a BCA assay kit (Beyotime Biotechnology Inc., China). Protein samples (40 μg protein per lane) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane for western blot analysis. The membrane was blocked for 1 h at room temperature (RT) and then incubated with a primary antibody overnight at 4 °C. We used the same anti−NF−κBp65 (AF5006, 1:750 dilution) and Lamin B1 (AF5161, 1:1000 dilution) antibodies (Affinity BioReagents, Golden, Colorado, USA) as those in our previous study [43]. Lamin B1 was used as control protein for nuclear protein. After being washed, the PVDF membrane was incubated for 1.5 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (A0208, 1:8000 dilution, Beyotime Biotechnology Inc., China) in tris-buffered saline with Tween (TBST). The immune complexes were visualised using electrochemiluminescence (ECL) reagents (Affinity Biosciences Inc., USA). The western blot bands were quantified using

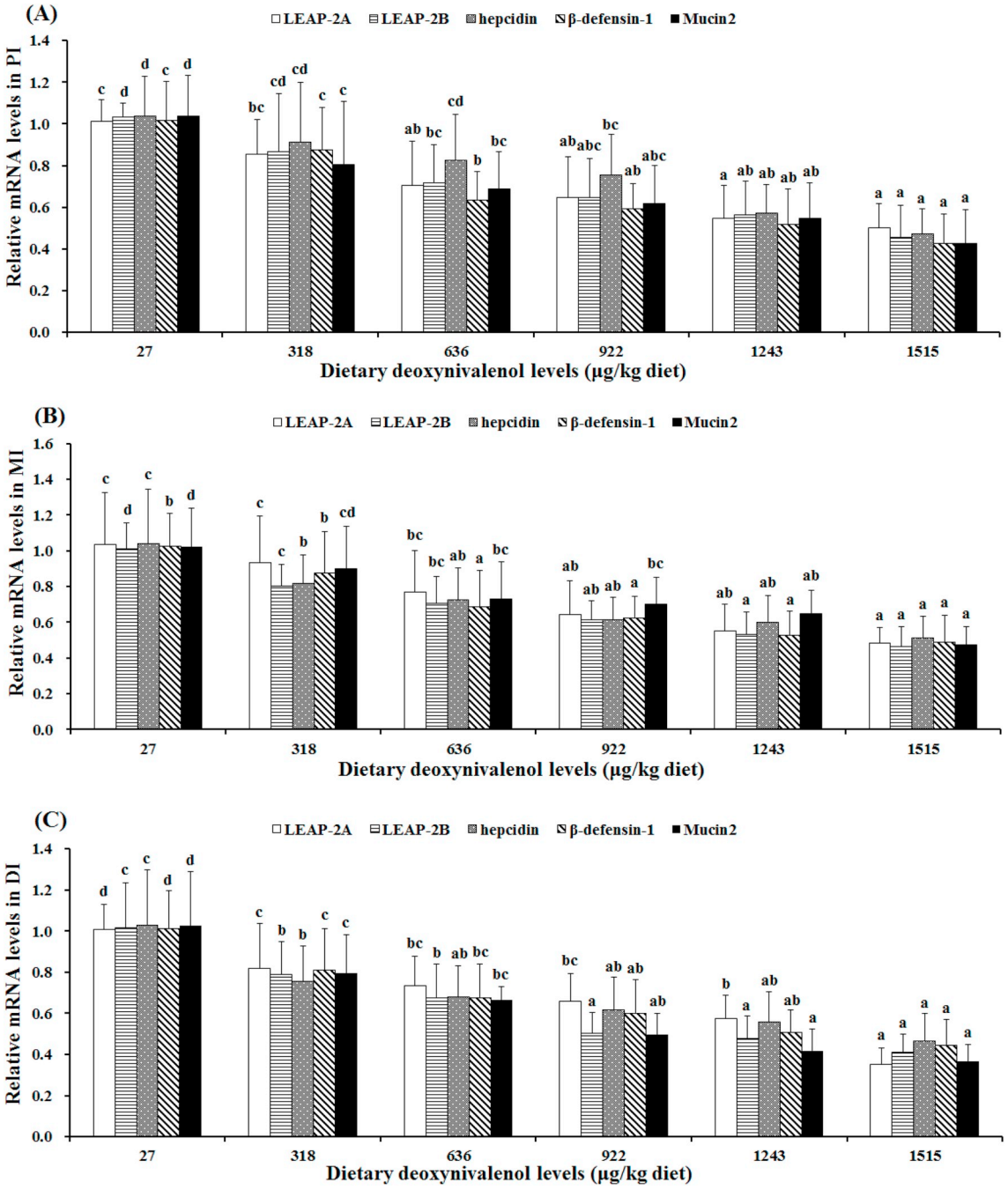

Fig. 2. Effects of graded levels of deoxynivalenol on relative mRNA levels of LEAP-2A, LEAP-2B, hepcidin, β-defensin-1 and Mucin2 in the PI (A), MI (B) and DI (C) of juvenile grass carp (*Ctenopharyngodon idella*). Data represent means (n = 6 individuals in each group), error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$).

NIH Image 1.63 software (National Institutes of Mental Health, Bethesda, USA). Different treatments were expressed relative to the level of the unsupplemented group. This experiment was repeated at least three times, and similar results were obtained each time.

2.8. Statistical analysis

Levene's test was used for the homogeneity of variances, and the Shapiro-Wilk test was used for normal distributions followed by one-way analysis of variance (ANOVA) with the Duncan's multiple-range test to evaluate significant differences among treatments at $P < 0.05$ with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The results were shown as the mean ± standard deviation (SD). Pearson correlation coefficient analysis was conducted using the Bivariate Correlation program in SPSS 18.0. The reasonable dose of DON for grass carp based on enteritis morbidity, and LZ and ACP activities as well as IgM content in the PI, were determined by broken-line model according to the method of Li et al. [44].

3. Results

3.1. Enteritis morbidity and intestinal histopathological lesions in juvenile grass carp after infection with A. hydrophila

Compared with the saline group, the *A. hydrophila* group showed obvious red and swollen phenomenon in the intestine of juvenile grass

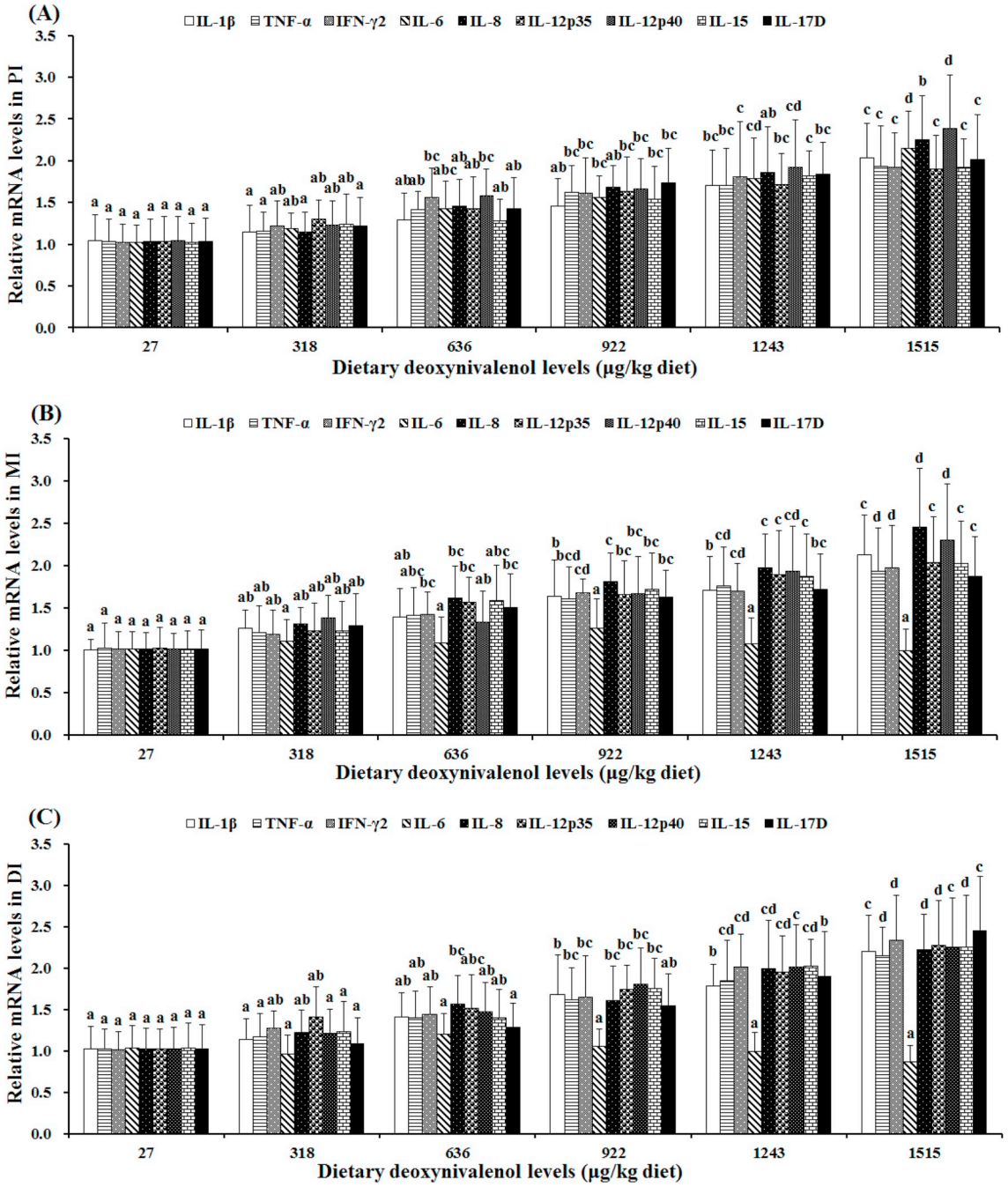

Fig. 3. Effects of graded levels of deoxynivalenol on relative mRNA levels of pro-inflammatory cytokines in the PI (A), MI (B) and DI (C) of juvenile grass carp (*Ctenopharyngodon idella*). Data represent means (n = 6 individuals in each group), error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$).

carp (supplementary materials Fig. S1). The obvious histopathological lesions caused by *A. hydrophila* were found in the three intestinal segments of juvenile grass carp (supplementary materials Fig. S2). In the PI, MI and DI, the oedema in the lamina propria was observed in the *A. hydrophila* group. In the PI of juvenile grass carp, we also found blood capillary hyperaemia in the *A. hydrophila* group. The enteritis morbidity of juvenile grass carp after infection with *A. hydrophila* is shown in Fig. 1. The enteritis morbidity of juvenile grass carp was increased in a dose-dependent manner. In comparison with the control group, the enteritis morbidity of juvenile grass carp was increased significantly with dietary DON levels up to 636 μg/kg diet ($P < 0.05$).

3.2. The immune response-related parameters in three intestinal segments of juvenile grass carp after infection with A. hydrophila

In comparison with the saline group, *A. hydrophila* significantly increased the activities of LZ and ACP and the content of C3, C4 and IgM in the PI, MI and DI of juvenile grass carp (supplementary materials Fig. S3). The effects of dietary DON on the LZ and ACP activities, C3, C4 and IgM content in three intestinal segments of juvenile grass carp are shown in Table 3. The activities of LZ and ACP, and the content of C3, C4 and IgM in the PI, MI, and DI of juvenile grass carp, were declining as dietary DON levels increased. In comparison with the control group, the activities of LZ and ACP, and the content of C3, C4 and IgM in the PI and MI of juvenile grass carp were all significantly lower with

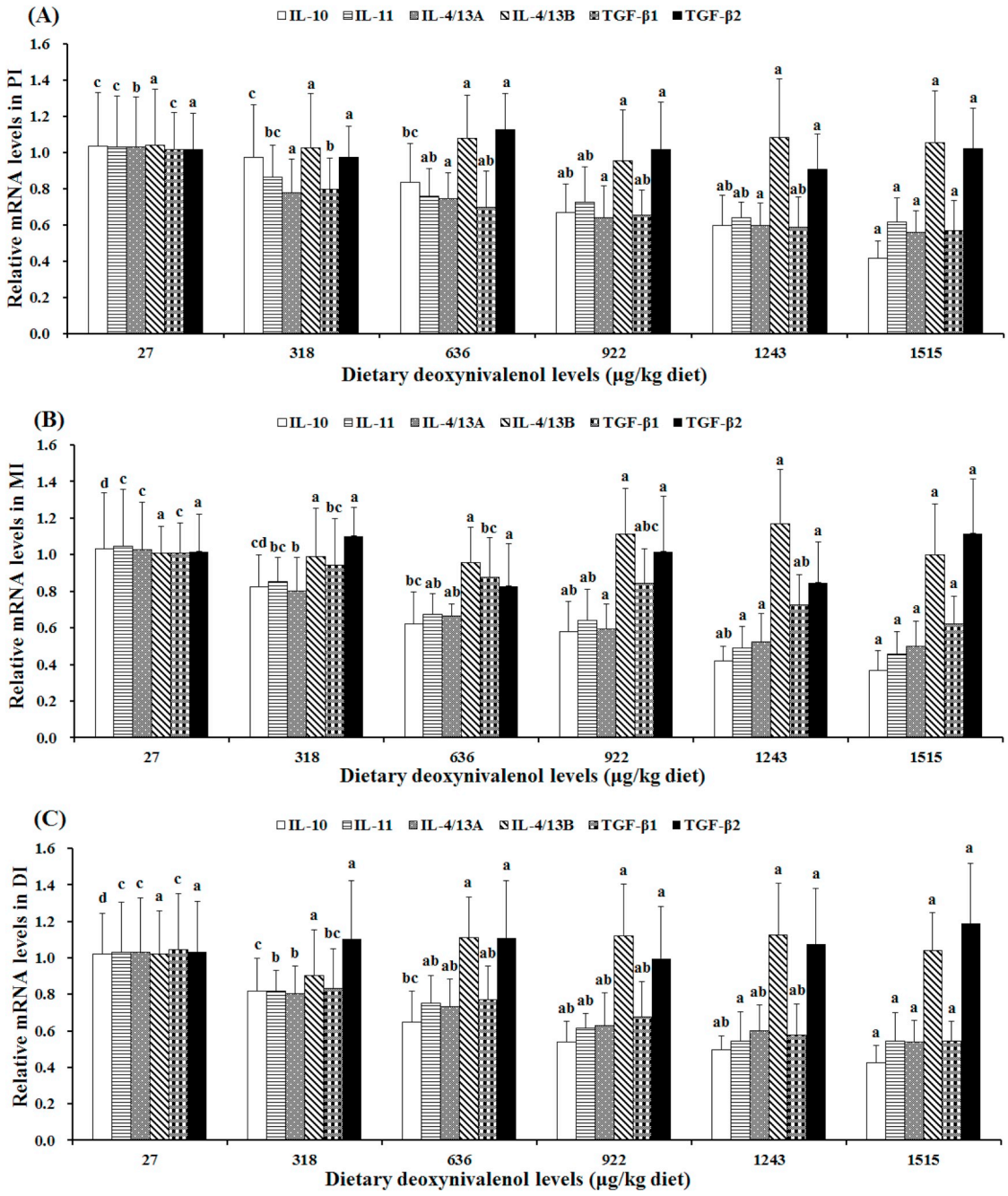

Fig. 4. Effects of graded levels of deoxynivalenol on relative mRNA levels of anti-inflammatory cytokines in the PI (A), MI (B) and DI (C) of juvenile grass carp (*Ctenopharyngodon idella*). Data represent means (n = 6 individuals in each group), error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$).

increasing dietary DON levels up to 636 μg/kg diet ($P < 0.05$). Additionally, the activities of LZ and ACP, and the content of C3, C4 and IgM in the DI of juvenile grass carp were decreased significantly as dietary DON levels up to 636, 636, 636, 636 and 1243 μg/kg diet ($P < 0.05$), respectively. The glucose content in the PI, MI and DI were significantly decreased with the dietary DON level up to 636, 318 and 636 μg/kg diet, respectively.

3.3. The relative mRNA levels of antimicrobial peptides (LEAP-2A, LEAP-2B, hepcidin and β-defensin-1) and mucin2 in three intestinal segments of juvenile grass carp after infection with A. hydrophila

The effects of dietary DON on the mRNA levels of antimicrobial peptides in three intestinal segments of juvenile grass carp are shown in Fig. 2. The mRNA levels of LEAP-2A, LEAP-2B, hepcidin, β-defensin-1 as well as mucin2 in the PI of juvenile grass carp, were significantly down-regulated with the increasing dietary DON levels up to 636, 636, 922, 636 and 318 μg/kg diet ($P < 0.05$), respectively. Similarly, the mRNA levels of LEAP-2A, LEAP-2B, hepcidin, β-defensin-1 as well as mucin2 in the MI of juvenile grass carp, were significantly down-regulated when the dietary DON levels reached 922, 318, 318, 636 and 636 μg/kg diet ($P < 0.05$), respectively. Additionally, as dietary DON levels increased to 318 μg/kg diet, the mRNA levels of LEAP-2A, LEAP-2B, hepcidin, β-defensin-1 and mucin2 in the DI of juvenile grass carp, were all significantly down-regulated ($P < 0.05$).

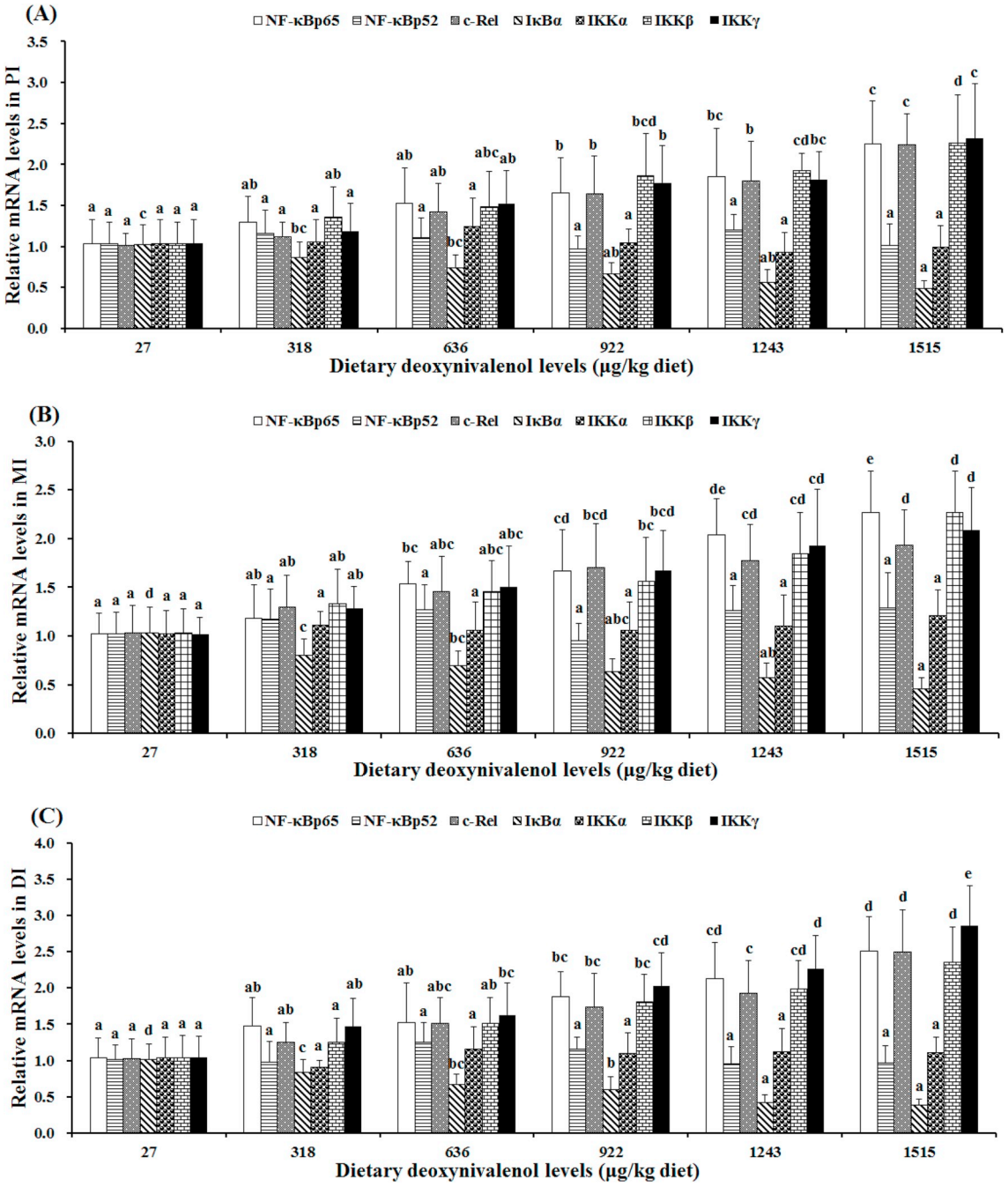

Fig. 5. Effects of graded levels of deoxynivalenol on relative mRNA levels of inflammation related signal molecules in the PI (A and D), MI (B and E) and DI (C and F) of juvenile grass carp (*Ctenopharyngodon idella*). Data represent means (n = 6 individuals in each group), error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$).

3.4. The relative mRNA levels of inflammatory cytokines in three intestinal segments of juvenile grass carp after infection with A. hydrophila

The effects of dietary DON on the mRNA levels of cytokines in three intestinal segments of juvenile grass carp are shown in Fig. 3 and Fig. 4. In the PI, the mRNA levels of IL-1β, TNF-α, IFN-γ2, IL-6, IL-8, IL-12p35, IL-12p40, IL-15 and IL-17D were significantly up-regulated as the increasing dietary DON levels reached 1243, 922, 636, 922, 1515, 922, 636, 922 and 922 μg/kg diet ($P < 0.05$), respectively. When the dietary DON levels increased to 922, 636, 318 and 318 μg/kg diet, the mRNA levels of IL-10, IL-11, IL-4/13A and TGF-β1 were significantly down-regulated ($P < 0.05$), respectively. In the MI, the mRNA levels of IL-1β, TNF-α, IFN-γ2, IL-8, IL-12p35, IL-12p40, IL-15 and IL-17D were significantly up-regulated with the increasing dietary DON levels up to 922, 922, 636, 636, 636, 922, 922 and 636 μg/kg diet ($P < 0.05$), respectively. As the dietary DON levels increased to 636, 636, 318 and 1243 μg/kg diet, the mRNA levels of IL-10, IL-11, IL-4/13A and TGF-β1 were significantly down-regulated ($P < 0.05$), respectively. In the DI, the mRNA levels of IL-1β, TNF-α, IFN-γ2, IL-8, IL-12p35, IL-12p40, IL-15 and IL-17D were significantly up-regulated when the increasing dietary DON levels reached 922, 922, 922, 636, 922, 922, 922 and 1243 μg/kg diet ($P < 0.05$), respectively. In addition, when the dietary DON levels increased to 318, 318, 318 and 636 μg/kg diet, the mRNA levels of IL-10, IL-11, IL-4/13A and TGF-β1 were significantly down-regulated ($P < 0.05$), respectively. Interestingly, dietary DON had no effects on the mRNA levels of IL-6 (in the MI and DI), and IL-4/13B and

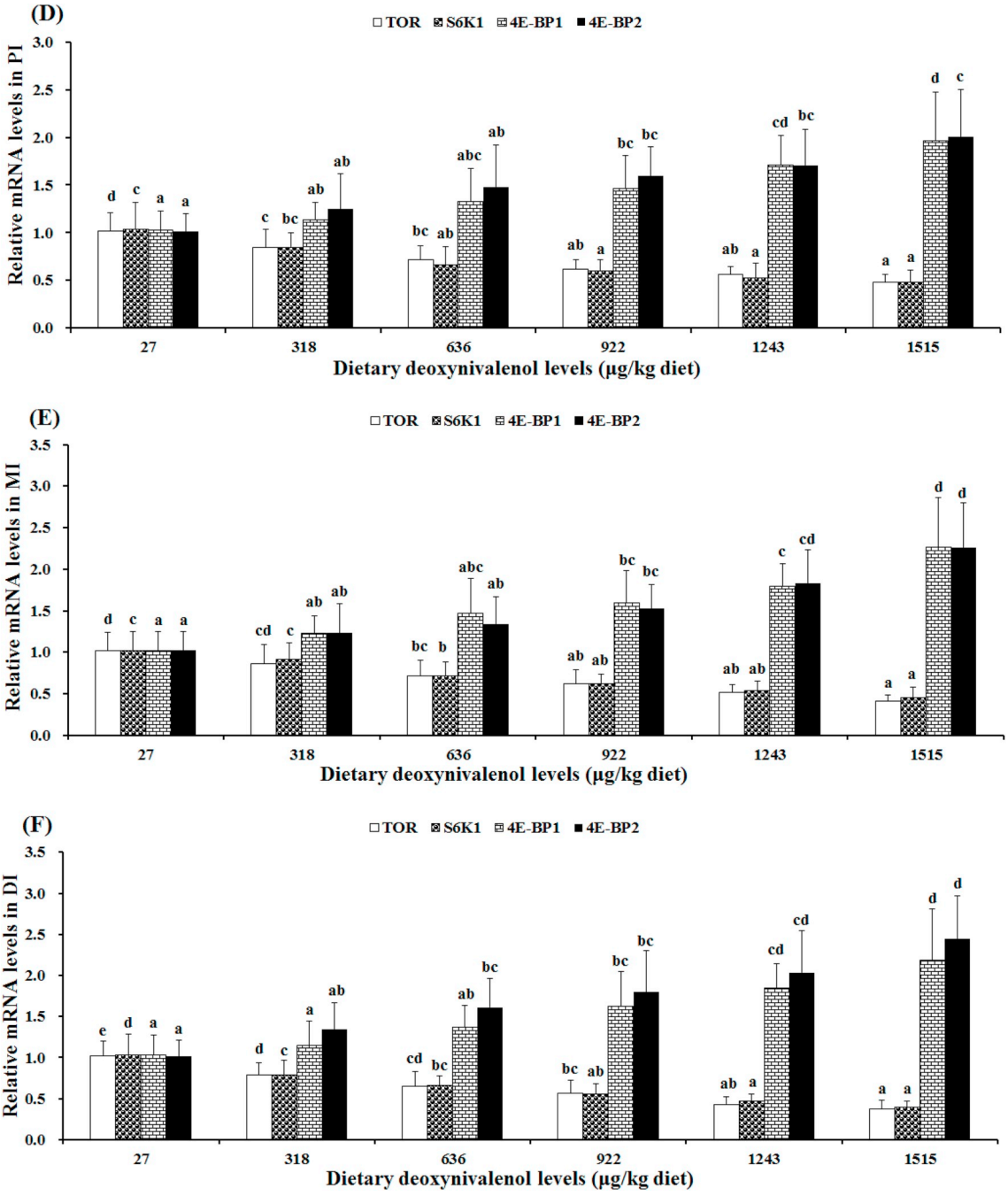

Fig. 5. (*continued*)

TGF-β2 in the PI, MI and DI of juvenile grass carp (P > 0.05).

3.5. The relative mRNA levels of inflammatory response-related signalling molecules in three intestinal segments of juvenile grass carp after infection with A. hydrophila

The effects of dietary DON on the mRNA levels of immune-related signalling molecules in three intestinal segments of juvenile grass carp are shown in Fig. 5. In the PI, the mRNA levels of NF-κBp65, c-Rel, IKKβ, IKKγ, 4E-BP1 and 4E-BP2 were all significantly up-regulated as the increasing dietary DON levels reached 922 μg/kg diet ($P < 0.05$). When the dietary DON levels increased to 922, 318 and 636 μg/kg diet, the mRNA levels of IκBα, TOR and S6K1 were significantly down-regulated ($P < 0.05$), respectively. In the MI, the mRNA levels of NF-κBp65, c-Rel, IKKβ, IKKγ, 4E-BP1 and 4E-BP2 were significantly up-regulated with the increasing dietary DON levels up to 636, 922, 922, 922, 922 and 922 μg/kg diet ($P < 0.05$), respectively. As the dietary DON levels increased to 318, 636 and 636 μg/kg diet, the mRNA levels of IκBα, TOR and S6K1 were significantly down-regulated ($P < 0.05$), respectively. In the DI, the mRNA levels of NF-κBp65, c-Rel, IKKβ, IKKγ, 4E-BP1 and 4E-BP2 were significantly up-regulated when the increasing dietary DON levels reached 922, 922, 922, 636, 922 and 636 μg/kg diet ($P < 0.05$), respectively. In addition, when the dietary DON levels increased to 318 μg/kg diet, the mRNA levels of IκBα, TOR and S6K1 were all significantly down-regulated ($P < 0.05$). Specifically, dietary DON had no effects on the mRNA levels of NF-κBp52 and IKKα in the PI, MI and DI of juvenile grass carp (P > 0.05).

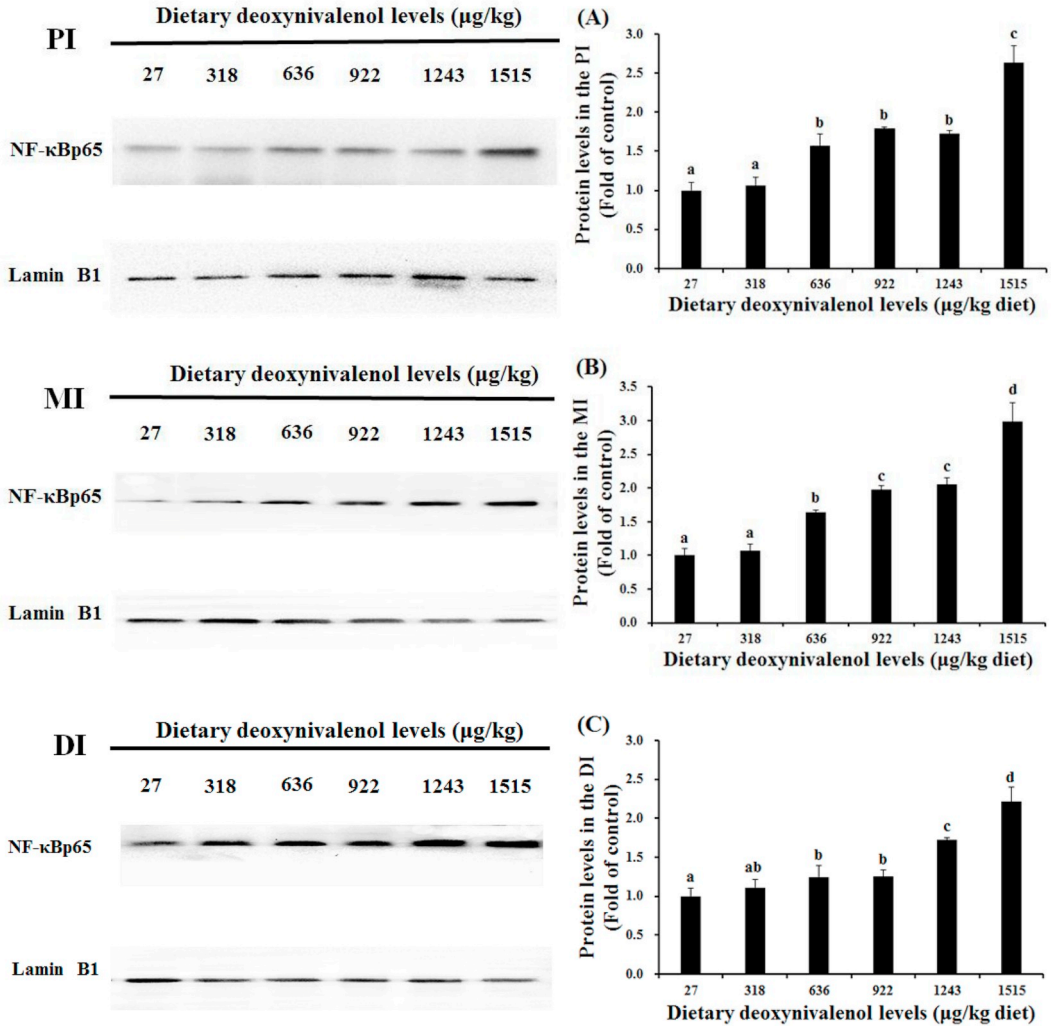

Fig. 6. Effects of graded levels of deoxynivalenol on protein levels of NF-κBp65 in the PI (A), MI (B) and DI (C) of juvenile grass carp (*Ctenopharyngodon idella*). Data represent means (n = 3 individuals in each group), error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$).

3.6. Protein levels of NF-κBp65 in the three intestinal segments of juvenile grass carp after infection with A. hydrophila

The effects of dietary DON on the protein levels of NF-κBp65 in the PI, MI and DI are shown in Fig. 6. In comparison with the control group, the protein levels of NF-κBp65 in the PI, MI and DI were increased significantly as dietary DON levels up to 636 μg/kg diet ($P < 0.05$).

3.7. Correlation analysis between the mRNA levels of inflammatory cytokines and the mRNA and protein levels of related signalling molecules in three intestinal segments of juvenile grass carp after infection with A. hydrophila

The correlation analysis showed that the mRNA levels of pro-inflammatory cytokines (except IL-6 in MI and DI) were positively related to NF-κBp65 protein levels and c-Rel mRNA levels, and the mRNA levels of IKKβ and IKKγ were negatively correlated with the mRNA levels of IκBα, which were negatively related to the protein levels of NF-κBp65 and the mRNA levels of c-Rel in three intestinal segments of juvenile grass carp (Table 4). Meanwhile, our results showed that the mRNA levels of anti-inflammatory cytokines IL-10, IL-11, IL-4/13A and TGF-β1 were positively related to the mRNA levels of TOR, and the mRNA levels of S6K1 were positively correlated with the mRNA levels of TOR, whereas the mRNA levels of 4E-BP1 and 4E-BP2 were negatively related to the mRNA levels of TOR in three intestinal segments of juvenile grass carp (Table 4).

4. Discussion

4.1. DON decreases innate and adaptive immune components in the intestine of fish under A. hydrophila infection

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 [45]. In the present study, DON levels up to 318 μg/kg diet decreased the LZ and ACP activities, as well as C3, C4 and IgM content, and down-regulated the mRNA levels of LEAP-2A, LEAP-2B, hepcidin, β-defensin-1 and mucin2 in the PI, MI and DI of juvenile grass carp after *A. hydrophila* infection. These data suggested that DON reduced intestinal immune function partly related to the decreased innate and adaptive immune components of fish. The negative effect of DON on intestinal immune function might be partly related to the inhibition of immune cell proliferation. In humans, DON has been reported to inhibit the cell proliferation of lymphocytes [46]. It is widely accepted that the immune function of fish is closely associated with lymphocytes, which are involved in the production of immune components [47]. Moreover, the intestines of fish showed mucosa-associated lymphoid tissues, which contains lymphocytes [48]. Hence, DON impaired fish intestinal immune function, which might be related to the inhibition of

Table 4
Correlation analysis of parameters in the PI, MI and DI.

Independent parameters	Dependent parameters	PI		MI		DI	
		Correlation coefficients	*P*	Correlation coefficients	*P*	Correlation coefficients	*P*
Nuclear NF-κB p65	IL-1β	+0.954	$P < 0.01$	+0.981	$P < 0.01$	+0.935	$P < 0.01$
	TNF-α	+0.953	$P < 0.01$	+0.960	$P < 0.01$	+0.947	$P < 0.01$
	IFN-γ2	+0.906	$P < 0.05$	+0.972	$P < 0.01$	+0.963	$P < 0.01$
	IL-6	+0.961	$P < 0.01$	−0.144	$P = 0.785$	−0.612	$P = 0.197$
	IL-8	+0.970	$P < 0.01$	+0.982	$P < 0.01$	+0.943	$P < 0.01$
	IL-12p35	+0.920	$P < 0.01$	+0.945	$P < 0.01$	+0.930	$P < 0.01$
	IL-12p40	+0.971	$P < 0.01$	+0.937	$P < 0.01$	+0.911	$P < 0.05$
	IL-15	+0.885	$P < 0.05$	+0.943	$P < 0.01$	+0.926	$P < 0.01$
	IL-17D	+0.925	$P < 0.01$	+0.928	$P < 0.01$	+0.983	$P < 0.01$
c-Rel	IL-1β	+0.991	$P < 0.01$	+0.973	$P < 0.01$	+0.994	$P < 0.01$
	TNF-α	+0.987	$P < 0.01$	+0.992	$P < 0.01$	+0.992	$P < 0.01$
	IFN-γ2	+0.956	$P < 0.01$	+0.991	$P < 0.01$	+0.989	$P < 0.01$
	IL-6	+0.995	$P < 0.01$	+0.106	$P = 0.841$	−0.502	$P = 0.310$
	IL-8	+0.999	$P < 0.01$	+0.977	$P < 0.01$	+0.976	$P < 0.01$
	IL-12p35	+0.968	$P < 0.01$	+0.983	$P < 0.01$	+0.984	$P < 0.01$
	IL-12p40	+0.994	$P < 0.01$	+0.950	$P < 0.01$	+0.978	$P < 0.01$
	IL-15	+0.961	$P < 0.01$	+0.990	$P < 0.01$	+0.978	$P < 0.01$
	IL-17D	+0.976	$P < 0.01$	+0.994	$P < 0.01$	+0.985	$P < 0.01$
TOR	IL-10	+0.962	$P < 0.01$	+0.991	$P < 0.01$	+0.991	$P < 0.01$
	IL-11	+0.991	$P < 0.01$	+0.988	$P < 0.01$	+0.990	$P < 0.01$
	IL-4/13A	+0.976	$P < 0.01$	+0.972	$P < 0.01$	+0.987	$P < 0.01$
	TGF-β1	+0.982	$P < 0.01$	+0.972	$P < 0.01$	+0.996	$P < 0.01$
	S6K1	+0.994	$P < 0.01$	+0.996	$P < 0.01$	+0.998	$P < 0.01$
	4E-BP2	−0.982	$P < 0.01$	−0.950	$P < 0.01$	−0.972	$P < 0.01$
IκBα	Nuclear NF-κB p65	−0.909	$P < 0.05$	−0.909	$P < 0.05$	−0.870	$P < 0.05$
	c-Rel	−0.962	$P < 0.01$	−0.983	$P < 0.01$	−0.940	$P < 0.01$
IKKβ	IκBα	−0.978	$P < 0.01$	−0.941	$P < 0.01$	−0.964	$P < 0.01$
IKKγ	IκBα	−0.965	$P < 0.01$	−0.977	$P < 0.01$	−0.953	$P < 0.01$

immune cell proliferation. However, further investigation should be conducted to verify this assumption.

4.2. DON aggravates inflammatory responses, which might be partly related to the NF-κB and TOR signalling pathways in the intestines of fish under A. hydrophila infection

Inflammatory cytokines are categorised into pro-inflammatory and anti-inflammatory cytokines [49]. A previous study reported that the up-regulation of pro-inflammatory cytokines (e.g., IL-1β) and the down-regulation of anti-inflammatory cytokines (e.g., TGF-β) could aggravate inflammatory responses in the Caco-2 cells [50]. Results from our study showed that compared with the control group, DON levels up to 318 μg/kg diet up-regulated the mRNA levels of major pro-inflammatory cytokines, and down-regulated the mRNA levels of main anti-inflammatory cytokines in the three intestinal segments of juvenile grass carp. These results indicate that DON could aggravate the intestinal inflammatory responses of fish.

Interestingly, our data showed that DON up-regulated the mRNA levels of IL-6 in the PI (rather than MI and DI), and down-regulated the mRNA levels of IL-4/13A (rather than IL-4/13B) and TGF-β1 (rather than TGF-β2) in the three intestinal segments of juvenile grass carp. These interesting phenomena might be explained as follows. First, DON only up-regulated IL-6 mRNA levels in PI (rather than MI and DI) of juvenile grass carp, which might be partly related to cholecystokinin (CCK). One report has shown that DON elevated the content of CCK in B6C3F1 mice [51], and CCK could increase IL-6 levels in the blood and liver of mice [52]. However, the expression of CCK in the PI was much higher than in the MI and DI of *S. prenanti* [53]. Hence, we suppose that DON up-regulated IL-6 mRNA levels in the PI (rather than MI and DI) partly due to the high expression of CCK in the PI rather than the MI and DI of fish. However, further investigation should be conducted to verify this assumption. In addition, DON down-regulated the mRNA levels of IL-4/13A (rather than IL-4/13B) in three intestinal segments of juvenile grass carp, which might be partly related to TOR and GATA3. The current study showed that DON down-regulated the mRNA levels of TOR in three intestinal segments of juvenile grass carp. Cook and Miller revealed that the absence of mTOR could down-regulate the protein expression of GATA3 in the T cells of mice [54]. It was reported that GATA3 could regulate the gene transcription of IL-4/13A through bounding with a TATA box [55], which existed in IL-4/13A (rather than IL-4/13B) of pufferfish (*Tetraodon nigroviridis*) [56]. Hence, we suppose that DON down-regulated TOR mRNA levels leading to the down-regulation of GATA-3, which could bind with the TATA box in the IL-4/13A gene (rather than IL-4/13B) and finally might result in the down-regulation of IL-4/13A (rather than IL-4/13B) mRNA levels in the intestine of fish. However, this underlying reason requires further study. Finally, DON up-regulated TGF-β1 (rather than TGF-β2) mRNA levels in the three intestinal segments of juvenile grass carp, which might be partly related to glucose. Weigert et al. [57] reported that glucose could up-regulate TGF-β1 (rather than TGF-β2) gene expression in the mesangial cells of human. Our results showed that increasing levels of DON in the feed induced a depression of glucose levels in the intestine of juvenile grass carp, which supported our hypothesis.

In addition, studies reported that the IKK complex (including IKKα, IKKβ and IKKγ) induce the phosphorylation of IκBα leading to their proteasome degradation and resulting in the activation of NF-κB (including NF-κBp65, NF-κBp52 and c-Rel), which play a vital role in the regulation of pro-inflammatory cytokine expression in mammalian cells [58]. In humans, the nuclear NF-κB protein level is considered a hallmark of the activation of the NF-κB signalling pathway [59]. Our results showed that DON activated the NF-κB signalling pathway by up-regulating NF-κBp65 protein levels in the three intestinal segments of juvenile grass carp. The correlation analysis showed that the mRNA levels

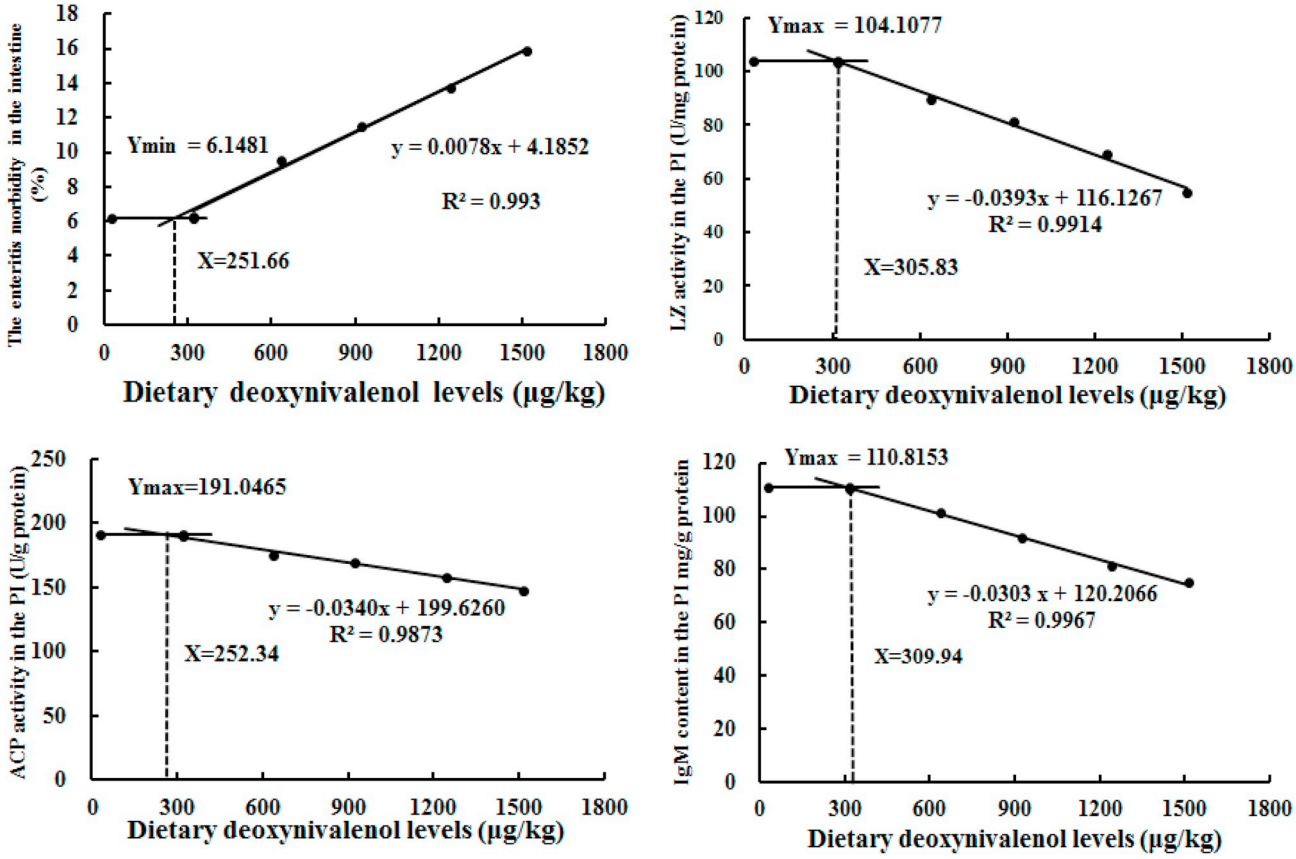

Fig. 7. Broken-line analysis of enteritis morbidity, and LZ and ACP activities as well as IgM content in the PI of juvenile grass carp containing graded levels of deoxynivalenol.

of pro-inflammatory cytokines (except IL-6 in MI and DI) were positively related to NF-κBp65 protein levels and c-Rel mRNA levels, and the mRNA levels of IKKβ and IKKγ were negatively correlated with the mRNA levels of IκBα, which were negatively related to the protein levels of NF-κBp65 and the mRNA levels of c-Rel in three intestinal segments of juvenile grass carp (Table 4). These data suggest that DON up-regulated pro-inflammatory cytokine mRNA levels partly relating to [(IKKβ and IKKγ)/IκBα/NF-κB (p65 and c-Rel)] signalling in the intestine of fish.

Interestingly, our results showed that DON up-regulated the mRNA levels of IKKβ and IKKγ (rather than IKKα), but did not have impact on the mRNA levels of NF-κBp52 in the three intestinal segments of juvenile grass carp. There are several reasons for these interesting phenomena as follows. First, DON up-regulated the mRNA levels of IKKβ and IKKγ (rather than IKKα) in three intestinal segments of juvenile grass carp, which might be partly related to TNF-α and protein kinase C-zeta (PKC-ζ). In the present study, DON up-regulated TNF-α mRNA levels in the three intestinal segments of juvenile grass carp. A study in rats reported that TNF-α induced PKC-ζ activity in C6 glioma cells [60], and PKC-ζ could up-regulate IKKβ and IKKγ (rather than IKKα) protein expression in rat Kupffer cells [61]. Hence, we suppose that DON up-regulated TNF-α mRNA levels leading to the increase in PKC-ζ activity, which resulted in the up-regulation of IKKβ and IKKγ (not IKKα) gene expression in the intestine of fish. However, this hypothesis requires further investigation. Second, DON did not have an effect on the mRNA levels of NF-κBp52 in three intestinal segments of juvenile grass carp, which might be partly related to the unaffected IKKα mRNA level. It has been confirmed that IKKα is crucial for the activation of NF-κBp52 in mammalian cells [62]. The present study showed that DON had no impact on the mRNA levels of IKKα in three intestinal segments of juvenile grass carp, which supported our hypothesis.

Furthermore, a study reported that mTOR could up-regulate S6K1 and down-regulate 4E-BP expression, which plays an important role in regulating the expression of anti-inflammatory cytokines in mammalian cells [63]. In this study, we investigated the effects of DON on TOR signalling in the intestine of fish for the first time, and the results showed that compared with the control group, DON levels up to 318 μg/kg diet down-regulated the mRNA levels of TOR and S6K1, and up-regulated the mRNA levels of 4E-BP1 and 4E-BP2 in three intestinal segments of juvenile grass carp. Further correlation analysis showed that the mRNA levels of anti-inflammatory cytokines IL-10, IL-11, IL-4/13A and TGF-β1 were positively related to the mRNA levels of TOR, and the mRNA levels of S6K1 were positively correlated with the mRNA levels of TOR, whereas the mRNA levels of 4E-BP1 and 4E-BP2 were negatively related to the mRNA levels of TOR in three intestinal segments of juvenile grass carp (Table 4). These data suggest that DON down-regulated the mRNA levels of anti-inflammatory cytokines partly relating to [TOR/(S6K1 and 4E-BP)] signalling in the intestine of fish.

4.3. The reasonable dose of DON for grass carp based on different indices

In our study, DON showed negative effects on the intestinal immune function of grass carp. Based on the enteritis morbidity, and LZ and ACP activity as well as IgM content in the PI, the reasonable dose of DON for grass carp were estimated to be 251.66, 305.83, 252.34 and 309.94 μg/kg diet, respectively (Fig. 7). These reasonable dose of DON for grass carp under *A. hydrophila* infection were lower than the reasonable dose of DON for grass carp form growth trial (452.37 μg/kg diet based on feed efficiency, using broken-line model to estimate again, the original reasonable dose is 318 μg/kg diet which was determined according to the method of Patterson et al.) reported by our previous study [7]. These results indicated that the grass carp under *A. hydrophila* infection

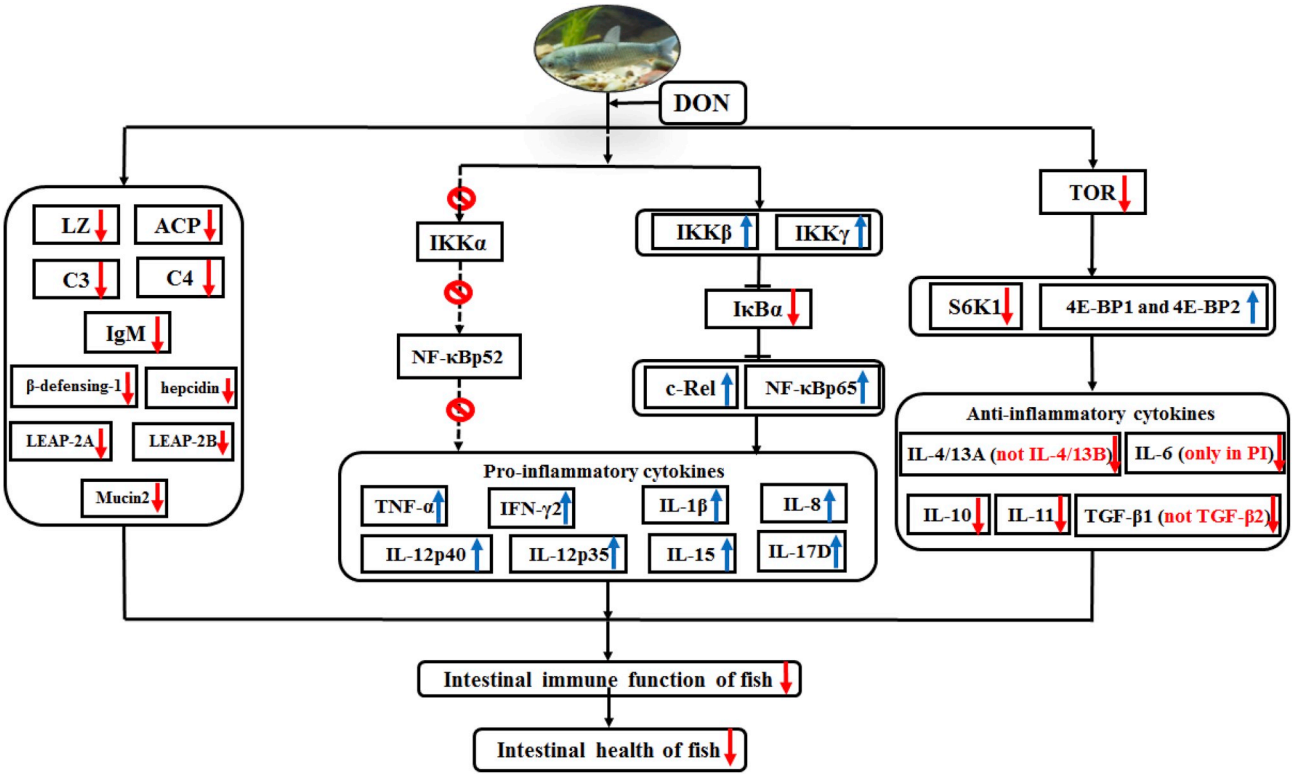

Fig. 8. The potential action pathways of dietary DON-disrupted intestinal immune function in fish.

were more sensitive to DON than the grass carp from the growth trial.

5. Conclusions

Taken together (Fig. 8), this study confirmed that DON could impair the intestinal immune function of fish as displayed in the following aspects. Compared with the control group, dietary DON levels up to 318 μg/kg diet reduced the content of innate and adaptive immune components and down-regulated the mRNA levels of all studied antimicrobial peptides and mucin2 in the intestine of fish, and aggravated inflammation by up-regulating the mRNA levels of main pro-inflammatory cytokines (except IL-6 in the MI and DI) and down-regulating the mRNA levels of major anti-inflammatory cytokines (except IL-4/13B and TGF-β2), which might be partly related to the NF-κB and TOR signalling pathways in the intestine of fish. Based on the enteritis morbidity and the LZ and ACP activities as well as IgM content in the PI, the reasonable doses of DON for grass carp were estimated to be 251.66, 305.83, 252.34 and 309.94 μg/kg 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.2018.10.039.

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