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Dietary iron deficiency impaired intestinal immune function of on-growing grass carp under the infection of *Aeromonas hydrophila*: Regulation of NF-κB and TOR signaling

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ABSTRACT

Iron is an important mineral element for fish. In this study, we investigated the influences of dietary iron deficiency on intestinal immune function as well as underlying signaling of on-growing grass carp (*Ctenopharyngodon idella*). Fish were fed with six graded level of dietary iron for sixty days, and a fourteen days' challenge test under infection of *Aeromonas hydrophila* thereafter. Results showed that compared with optimal iron level, iron deficiency increased enteritis morbidity, decreased lysozyme (LZ) and acid phosphatase (ACP) activities, complement 3 (C3), C4 and immunoglobulin M (IgM) concentrations and down-regulated mRNA levels of hepcidin, liver expressed antimicrobial peptide 2A (LEAP-2A), LEAP-2B, Mucin2, β-defensin-1, anti-inflammatory cytokines transforming growth factor β1 (TGF-β1), TGF-β2, interleukin 4/13A (IL-4/13A), IL-4/13B, IL-10, IL-11 and IL-15, inhibitor of κBα (IκBα), target of rapamycin (TOR) and ribosomal protein S6 kinase 1 (S6K1), whereas up-regulated mRNA levels of pro-inflammatory cytokines IL-1β, interferon γ2 (IFN-γ2), IL-8, IL-12p35, IL-12p40 and IL-17D, nuclear factor kappa B (NF-κB) p65, IκB kinases α (IKKα), IKKβ and eIF4E-binding protein (4E-BP) in intestine of on-growing grass carp, indicating that iron deficiency impaired intestinal immune function of fish under infection of *A. hydrophila*. Besides, iron excess also increased enteritis morbidity and impaired immune function of fish under infection of *A. hydrophila*. In addition, the effect of ferrous fumarate on intestinal immune function of on-growing grass carp is more efficient than ferrous sulfate. Finally, based on ability against enteritis, LZ activities in mid intestine and distal intestine, we recommended adding 83.37, 86.71 and 85.39 mg iron/kg into diet, respectively.

1. Introduction

Nowadays, farmed fish are more susceptible to diseases due to the intensive aquaculture, especially enteritis, which leads to decreased immunity and high mortality [1]. Iron is an essential mineral element for fish growth [2]. It has been reported that iron play important roles in immune function of fish [3].Study revealed that dietary iron deficiency led to decreased disease resistance and immune function of fish [4–7]. Also, our previous studies have proved that iron deficiency resulted in decreased bacterial resistance and impaired immune function in head kidney, spleen, skin and gill of on-growing grass carp (*Ctenopharyngodon idellus*) [8,9]. According to our previous study, the influences of iron on immune function of various organs seems different, in which dietary iron deficiency inhibited IKKβ/NF-κB p65 signaling in head kidney, spleen and skin of on-growing grass carp, whereas inhibited IKKα, β, γ/NF-κB p65 signaling in gill [8,9]. However, up to now, there is no study investigating the influences of dietary iron deficiency on intestinal immune function of fish, which requires investigation.

Intestine is an important mucosal immune organ of fish [10]. Usually, intestinal immune function in fish is tightly associated with antibacterial compounds, antibacterial peptides and cytokines [11–14].

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Furthermore, study revealed that in humans, target of nuclear factor-κB (NF-κB) signaling and mammalian rapamycin (mTOR) signaling regulated cytokines transcription [15,16]. It has been reported that lactoferrin, an iron binding protein, up-regulated mRNA level of lysozyme (LZ) in small intestine of mice [17] and increased immunoglobulin concentration in intestinal fluid of mice [18]. In the study of Caco-2 human intestinal cells, depletion of iron down-regulated expression of transforming growth factor β (TGF-β) [19]. Moreover, study revealed that depletion of iron enhanced phosphorylation of NF-κBp65 in Caco-2 cells [20]. Besides, iron deficiency reduced phosphorylation of mTOR substrates in Caco-2 cells [21]. These data suggest that iron might influence intestinal antibacterial compounds, antibacterial peptides and cytokines, which requires investigation.

Aeromonas hydrophila is common in freshwater which causes intestinal inflammation and impaired immune function in fish [22]. Yang et al. (2017) [23] have reported that under infection of *A. hydrophila*, the mRNA levels of antibacterial peptide defensin and hepcidin were up-regulated. Besides, in the study of Song et al. (2014) [24], the mRNA levels of interleukin (IL) -1β, IL-8 and tumor necrosis factor α (TNF-α) were up-regulated. Study revealed that iron play important roles in bacterial infection [25]. However, there is no study investigating the effects of dietary iron on intestinal immune function after challenge with *A. hydrophila*. Hence, we investigated the influences of iron on antibacterial compounds, antibacterial peptides and cytokines, as well as the possible signaling in the intestine of on-growing grass carp after challenge with *A. hydrophila*, which might provide basic data for better understanding of the influences of dietary iron on intestinal immune function of fish. Meanwhile, dietary iron requirement for grass carp based on different indices (under the basis of ferrous fumarate) might provide a practical evidences for development of aquaculture.

2. Materials and methods

2.1. Experimental diets preparation

As shown in Table 1, formulation of the basal diet is similar with our previous study [9]. Six experiment diets with different iron level [ferrous fumarate (FF, $C_4H_2FeO_4$) as iron sources] (basal diet, 35, 60, 85, 110 and 135 mg iron/kg diet) was used. The final iron concentrations of the six diets were 12.15 (basal diet), 35.38, 63.47, 86.43, 111.09 and 136.37 mg/kg diet, as determined by atomic absorption spectrometry according to AOAC (2000). Moreover, **ferrous sulfate** was used in diet seven to provide 73.50 mg iron/kg, which was the positive control in this study. The prepared diets were stored at −20 °C according to Ling et al. (2010) [26].

2.2. Feeding trial

The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. Grass carp were purchased from fishery (Sichuan, China). Prior to the experiment, fish were acclimatized to the experimental environment for four weeks, according to Ni et al. (2016) [27]. 540 fish (mean weight 242 ± 0.580 g) were assigned to eighteen experimental cages (1.4 L × 1.4 W × 1.4 H m) randomly, resulting in thirty fish per cage. The cages were placed in culture pond of 9750 m^3 water capacity. Rates of water flow were adjusted and oxygen was enhanced by micropore aeration continuously in the bottom. Feeding trial lasted for sixty days. Six respective diets were hand-fed to fish to satiation fourth daily. After 30 min, remaining feeds were gathered through the disc equipped at 100 cm diameter in the bottom according to Xu et al. (2016) [28]. During experimental period, pH value, water temperature, dissolved oxygen and other procedures were same with our previous study [8,9]. Water samples were gathered every day and iron concentration in the water was determined to be 0.034 ± 0.010 mg/L according to AOAC (2000).

Table 1
Composition and nutrients content of basal diet.

Ingredients	%	Nutrients content	%
Fish meal	3.00	Crude protein[d]	30.30
Casein	24.25	Crude lipid[d]	5.22
Gelatin	8.58	n-3[e]	1.04
DL-Met (99%)	0.28	n-6[e]	0.96
L-Trp (99%)	0.09	Available phosphorus[f]	0.40
α-starch	24.00		
Corn starch	23.63		
Fish oil	2.77		
Soybean oil	1.80		
Cellulose	5.00		
$Ca(H_2PO_4)_2$	1.55		
Vitamin premix[a]	1.00		
Mineral premix[b]	2.00		
Iron premix[c]	1.00		
Choline chloride (50%)	1.00		
Ethoxyquin (30%)	0.05		

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

[b] Mineral premix (g/kg premix): $MnSO_4·H_2O$ (31.8% Mn), 2.6590 g; $MgSO_4·H_2O$ (15.0% Mg), 200.0000 g; $ZnSO_4·H_2O$ (34.5% Zn), 8.2460 g; $CuSO_4·5H_2O$ (25.0% Cu), 0.9560 g; KI (76.9% I), 0.0650 g; Na_2SeO_3 (44.7% Se), 0.0168 g. All ingredients were diluted with corn starch to 1 kg.

[c] Iron premix: diet 2–6 were added with ferrous fumarate and diet 7 was added with ferrous sulfate.

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

[e] n-3 and n-6 contents were calculated according to Zeng et al. (2016) [65].

[f] Available phosphorus were calculated according to Wen et al. (2015) [66].

2.3. Challenge test and sample collection

After feeding trial, we conducted a challenge test through *A. hydrophila* infection. The *A. hydrophila* was provided by College of Veterinary Medicine, Sichuan Agricultural University. Fifteen fish randomly acquired from each treatment were intraperitoneal injected with 1.0 ml A. *hydrophila* [(concentration of 2.5 × 10^9 colony-forming units (CFU)/ml]. The injected bacterial concentration activated the immune system and consequently enable the investigation of effluent on reactivity against a threatening disease without killing fish according to our preliminary study data (unpublished data). The challenge test lasted for 14 days, according to our previous study [29]. During the challenge test, the experimental conditions, including experimental diets, were as same as the feeding trial. At the end of the challenge test, we conducted a scoring system to evaluate the severity of intestine inflammation, according to Song et al. (2014) [24].

After scoring, all fish were removed from the cages and then anaesthetized in a benzocaine bath as described by Geraylou et al. (2013) [30]. Next, the intestinal samples were quickly removed, segmented [proximal intestine (PI), mid intestine (MI) and distal intestine (DI)], and frozen in liquid nitrogen, and then transferred to −80 °C refrigerator until use [28]. The intestines were homogenized on ice in 10 vol (w/v) of ice-cold physiological saline and centrifuged at 6000 *g* at 4 °C for 20 min, then the collected supernatant was stored for the analysis of immune function related parameters [31]. LZ and acid phosphatase (ACP) activities were measured as described by Wu et al. (2013) [32]. Complement 3 (C3), C4 and immunoglobulin M (IgM) concentrations were measured as described by Pan et al. (2016) [33].

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

Total six RNA samples per treatment were isolated from intestine using RNAiso Plus Kit (Takara, Dalian, China), and the RNA was dissolved in DEPC treated-water and quantified by spectrophotometry at 260 nm and 280 nm according to Camoglio et al. (2002) [34]. Then we used agarose gel (1%) electrophoresis to assess the quality, as described by Pan et al. (2016) [33]. Single-stranded cDNA was prepared from total RNA by reverse transcription using a PrimeScripte RT reagent Kit, according to the manufacturer's protocols. Primers used to amplify the target genes are listed in Table 2. Real-time PCR assays were launched in a quantitative thermal cycler (Prism 7900HT, ABI, USA). Melt curve analysis was launched after amplification to confirm the specificity and purity of all PCR products. β-actin was used as an endogenous reference to normalize the template amount based on the results of our preliminary experiment concerning the evaluation of internal control genes (data not shown). The target and housekeeping gene amplification efficiency were calculated according to the specific gene standard curves generated from 10-fold serial dilutions. Results of gene expression were analyzed using the $2^{-\Delta\Delta CT}$ method according to Tang et al. (2013) [35].

2.5. Calculations and statistical analysis

Statistical analysis was performed using SPSS 25.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2016. One-way ANOVA model was used for data analysis, and Duncan's multiple range test was used to compare means when significant differences ($P < 0.05$) were found, as described by Chen et al. (2015) [36]. Quadratic regression model was used to estimate the optimal dietary iron requirement for on-growing grass carp according to Zhang et al. (2016) [37].

3. Results

3.1. Enteritis morbidity of on-growing grass carp

The enteritis morbidity (Fig. 1) under infection of *A. hydrophila* decreased first and then increased with dietary iron level increases, and the morbidity is lowest in 63.47 mg iron/kg diet ($P < 0.05$). Compared with the optimal iron level, iron deficiency led to an obvious enteritis symptom (Fig. 2).

3.2. Antibacterial compounds in the intestine of on-growing grass carp

As shown in Table 3 and Fig. 3. **In proximal intestine (PI)**, Activitiy of LZ and concentrations of C4 and IgM were highest in 86.43 mg iron/kg diet ($P < 0.05$). With iron level up to 63.47 and 86.43 mg/kg, activity of ACP and concentration of C4 increased first, and then decreased. With dietary iron level up to 63.47 mg/kg, mRNA levels of hepcidin, LEAP-2A, LEAP-2B, Mucin2 and β-defensin-1 up-regulated first, and then down-regulated. **In mid intestine (MI)**, concentrations of C3 and C4 were highest in 86.43 mg iron/kg diet ($P < 0.05$). Activities of LZ and ACP, and concentration of IgM increased with dietary iron level up to 63.47, 86.43 and 86.43 mg/kg, respectively, and then decreased. With dietary iron level up to 63.47 mg/kg, mRNA levels of LEAP-2A, LEAP-2B, Mucin2 and β-defensin-1 up-regulated first, and

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

Target gene	Primer sequence Forward (5'→3')	Primer sequence Reverse (5'→3')	Temperature (°C)	Accession number
hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59.3	JQ246442.1
LEAP-2A	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59.3	FJ390414
LEAP-2B	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59.3	KT625603
β-defensin-1	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58.4	KT445868
TNF-α	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC	58.4	HQ696609
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	57.1	JQ692172
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	60.4	JX657682
IL-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	62.3	KC535507.1
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	60.3	JN663841
IL-12p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55.4	KF944667.1
IL-12p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	59.0	KF944668.1
IL-17D	GTGTCCAGGAGAGCACCAAG	GCGAGAGGCTGAGGAAGTTT	62.3	KF245426.1
TGF-β1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	55.9	EU099588
TGF-β2	TACATTGACAGCAAGGTGGTG	TCTTGTTGGGGATGATGTAGTT	55.9	KM279716
IL-4/13A	CTACTGCTCGCTTTCGCTGT	CCCAGTTTTCAGTTCTCTCAGG	55.9	KT445871
IL-4/13B	TGTGAACCAGACCCTACATAACC	TTCAGGACCTTTGCTGCTTG	55.9	KT625600
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	61.4	HQ388294
IL-11	GGTTCAAGTCTCTTCCAGCGAT	TGCGTGTTATTTTGTTCAGCCA	57.0	KT445870
IL-15	CCTTCCAACAATCTCGCTTC	AACACATCTTCCAGTTCTCCTT	61.4	KT445872
NF-κBp65	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	62.3	KJ526214
NF-κBp52	TCAGTGTAACGACAACGGGAT	ATACTTCAGCCACACCTCTCTTAG	58.4	KM279720
IκBα	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA	62.3	KJ125069
IKKα	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA	60.3	KM279718
IKKβ	GTGGCGGTGGATTATTGG	GCACGGGTTGCCAGTTTG	60.3	KP125491
IKKγ	AGAGGCTCGTCATAGTGG	CTGTGATTGGCTTGCTTT	58.4	KM079079
TOR	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	61.4	JX854449
S6K1	TGGAGGAGGTAATGGACG	ACATAAAGCAGCCTGACG	54.0	EF373673
4E-BP1	GCTGGCTGAGTTTGTGGTTG	CGAGTCGTGCTAAAAAGGGTC	60.3	KT757305
4E-BP2	CACTTTATTCTCCACCACCCC	TTCATTGAGGATGTTCTTGCC	60.3	KT757306
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	61.4	M25013

[a] LEAP-2, liver expressed antimicrobial peptide 2; 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 kinase 1; 4E-BP, eIF4E-binding protein.

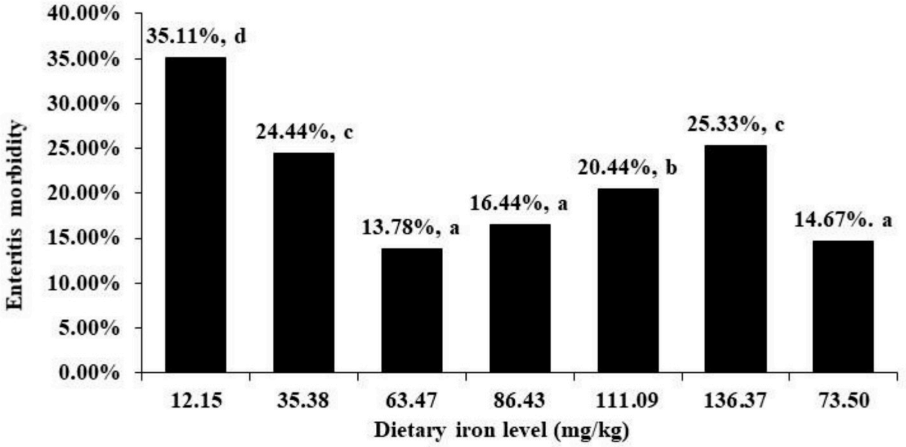

Fig. 1. Effects of dietary iron level (mg/kg diet) on enteritis morbidity of on-growing grass carp (*Ctenopharyngodon idella*) under infection of *A. hydrophila*. Data represent means of fifteen fish in each group. Values having different letters are significantly different ($P < 0.05$).

then down-regulated. Besides, mRNA level of hepcidin up-regulated with dietary iron level up to 86.43 mg/kg, and then down-regulated. **In distal intestine (DI)**, Activity of ACP and concentration of C3 were highest in 86.43 mg iron/kg diet ($P < 0.05$). With dietary iron level up to 86.43, 86.43 and 63.47 mg/kg diet, respectively, activity of LZ and concentrations of C4 and IgM increased, and then decreased. In addition, with dietary iron level up to 63.47 mg/kg, mRNA levels of hepcidin, LEAP-2A, LEAP-2B, Mucin2 and β-defensin-1 up-regulated first, and then down-regulated (See Table 3).

3.3. Inflammation related parameters in the intestine of on-growing grass carp

As shown in Figs. 4–6. **In PI**, mRNA levels of IL-8, IL-12p40, IκB kinase β (IKKβ), IKKγ and eIF4E-binding proteins 2 (4E-BP2) down-regulated with dietary iron level up to 63.47 mg/kg, and then up-regulated. Besides, with dietary iron level up to 63.47 mg/kg, mRNA levels of TGF-β1, TGF-β2, IL-4/13B, IL-10, IL-11, IL-15 and TOR up-regulated first, and then down-regulated. With dietary iron level up to 86.43 mg/kg, mRNA levels of interferon γ2 (IFN-γ2), IL-1β, IL-12p35, IL-17D, NF-κBp65 and 4E-BP1 down-regulate first, and then up-regulated. And with dietary iron level up to 86.43 mg/kg, mRNA levels of IL-4/13A, inhibitor of κBα (IκBα) and ribosomal protein S6 kinases 1 (S6K1) up-regulated first and then down-regulated. However, dietary iron level had no influences on mRNA levels of TNF-α, IL-6, NF-κBp52 and IKKα in PI of on-growing grass carp.

In MI, with dietary iron level up to 63.47 mg/kg, mRNA levels of TNF-α, IL-1β, IFN-γ2, IL-8, IL-12p35, IL-12p40, NF-κBp65, IKKβ, and 4E-BP2 down-regulated first, and then up-regulated. Besides, mRNA levels of TGF-β2, IL-4/13A and S6K1 up-regulated first with dietary iron level up to 63.47 mg/kg, and then down-regulated. With dietary iron level up to 86.43 mg/kg, mRNA levels of IL-17D, IKKγ and 4E-BP1 down-regulated first and then up-regulated. In addition, mRNA levels of TGF-β1, IL-4/13B, IL-10, IL-11, IL-15, IκBα and TOR up-regulated first, and then down-regulated. However, dietary iron level had no influences on mRNA levels of IL-6, NF-κBp52 and IKKα in MI of on-growing grass carp.

In DI, with dietary iron level up to 63.47 mg/kg, mRNA levels of TNF-α, IL-1β, IL-8, IL-12p40, IKKβ and 4E-BP2 down-regulated first, and then up-regulated. And with dietary iron level up to 63.47 mg/kg, mRNA levels of TGF-β1, TGF-β2, IL-4/13B, IL-10, IL-11, IL-15 and TOR up-regulated first, and then down-regulated. In addition, with dietary iron level up to 86.43 mg/kg, mRNA levels of IFN-γ2, IL-12p35, IL-17D, NF-κBp65, IKKγ and 4E-BP1 down-regulated first, and then up-regulated. With dietary iron level up to 86.43 mg/kg, mRNA levels of IL-4/13A, IκBα and S6K1 up-regulated first, and then down-regulated. However, dietary iron had no influences on mRNA levels of IL-6, NF-κBp52 and IKKα in DI of on-growing grass carp.

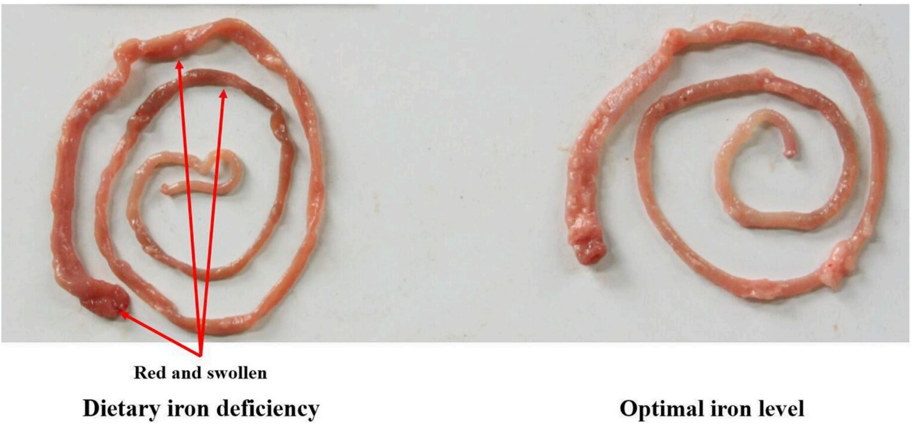

Fig. 2. Compared with optimal iron level, dietary iron deficiency led to obviously enteritis in on-growing grass carp (*Ctenopharyngodon idella*) under infection of *A. hydrophila*.

Table 3
Effects of dietary iron level on immune parameters in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of on-growing grass carp (*Ctenopharyngodon idella*) under infection of *Aeromonas hydrophila*[a].

	Ferrous fumarate						Ferrous sulfate
	Dietary iron level (mg/kg diet)						
	12.15	**35.38**	**63.47**	**86.43**	**111.09**	**136.37**	**73.50**
PI							
LZ	45.79 ± 1.28^{a}	70.28 ± 21.97^{b}	91.85 ± 20.14^{c}	123.28 ± 6.75^{d}	103.47 ± 20.43^{cd}	91.11 ± 4.54^{c}	103.20 ± 26.60^{cd}
ACP	79.29 ± 6.43^{a}	115.03 ± 8.30^{b}	175.59 ± 15.92^{c}	168.15 ± 12.08^{c}	163.47 ± 11.58^{c}	122.41 ± 5.58^{b}	169.05 ± 14.01^{c}
C3	18.50 ± 1.73^{a}	22.57 ± 1.62^{b}	26.95 ± 1.63^{d}	28.06 ± 2.01^{d}	24.64 ± 1.18^{c}	20.69 ± 1.77^{b}	26.23 ± 2.03^{cd}
C4	4.16 ± 0.27^{a}	5.74 ± 0.47^{b}	7.52 ± 0.48^{c}	8.43 ± 0.72^{d}	6.96 ± 0.67^{c}	5.97 ± 0.44^{b}	7.55 ± 0.55^{c}
IgM	52.47 ± 4.44^{a}	57.77 ± 4.28^{ab}	62.68 ± 4.06^{b}	68.63 ± 4.41^{c}	59.68 ± 5.38^{b}	57.18 ± 4.55^{ab}	61.42 ± 2.48^{b}
MI							
LZ	123.54 ± 21.58^{a}	164.49 ± 11.91^{b}	207.09 ± 22.51^{d}	192.55 ± 12.02^{cd}	176.04 ± 9.15^{bc}	175.33 ± 23.71^{bc}	188.75 ± 18.68^{cd}
ACP	176.24 ± 14.29^{a}	237.78 ± 15.54^{b}	274.15 ± 25.49^{bc}	280.92 ± 21.66^{c}	264.31 ± 7.75^{bc}	258.29 ± 10.38^{c}	271.25 ± 19.77^{bc}
C3	19.66 ± 0.66^{a}	23.62 ± 1.58^{b}	27.74 ± 1.82^{c}	32.99 ± 2.01^{d}	27.53 ± 2.61^{c}	25.78 ± 2.26^{c}	30.98 ± 1.08^{d}
C4	4.94 ± 0.30^{a}	5.65 ± 0.50^{b}	6.43 ± 0.32^{cd}	7.77 ± 0.65^{e}	6.36 ± 0.30^{cd}	6.08 ± 0.49^{bc}	6.93 ± 0.56^{d}
IgM	59.68 ± 4.94^{a}	64.31 ± 3.24^{ab}	68.17 ± 4.04^{bc}	71.15 ± 6.56^{c}	65.02 ± 5.63^{abc}	62.00 ± 4.25^{ab}	66.28 ± 5.26^{bc}
DI							
LZ	142.08 ± 8.68^{a}	202.74 ± 23.83^{b}	271.95 ± 18.28^{de}	282.16 ± 25.67^{e}	242.59 ± 31.72^{cd}	214.75 ± 27.82^{bc}	266.82 ± 30.86^{de}
ACP	167.25 ± 10.98^{a}	228.77 ± 14.37^{b}	360.10 ± 19.81^{e}	391.07 ± 37.18^{f}	312.29 ± 20.30^{d}	281.80 ± 19.19^{c}	343.96 ± 25.72^{e}
C3	23.10 ± 1.77^{a}	25.63 ± 1.76^{b}	29.19 ± 2.02^{cd}	32.79 ± 2.60^{e}	28.61 ± 1.59^{c}	26.79 ± 2.32^{bc}	31.19 ± 2.08^{de}
C4	6.39 ± 0.43^{a}	7.61 ± 0.26^{bc}	8.04 ± 0.56^{cd}	8.60 ± 0.79^{d}	7.52 ± 0.55^{bc}	7.20 ± 0.30^{bc}	7.98 ± 0.57^{cd}
IgM	54.80 ± 4.94^{a}	59.39 ± 5.18^{ab}	66.24 ± 3.54^{c}	63.70 ± 5.69^{bc}	60.91 ± 5.06^{bc}	58.04 ± 2.92^{ab}	63.25 ± 4.72^{bc}

Regression equation	Regression coefficient	***P***
PI		
$Y_{LZ} = -0.0101 \times ^{2} + 1.9001x + 20.3059$	0.9177	$P < 0.05$
$Y_{ACP} = -0.0187 \times ^{2} + 3.1791x + 38.3120$	0.9535	$P < 0.01$
$Y_{C3} = -0.0021 \times ^{2} + 0.3263x + 14.4359$	0.9718	$P < 0.01$
$Y_{C4} = -0.0007 \times ^{2} + 0.1262x + 2.5589$	0.9446	$P < 0.05$
$Y_{IgM} = -0.0027 \times ^{2} + 0.4439x + 46.7327$	0.8234	$P = 0.074$
MI		
$Y_{ACP} = -0.0157 \times ^{2} + 2.9002x + 148.5650$	0.9610	$P < 0.01$
$Y_{C3} = -0.0019 \times ^{2} + 0.3400x + 15.0912$	0.8602	$P = 0.052$
$Y_{C4} = -0.0004 \times ^{2} + 0.0690x + 3.9727$	0.7769	$P = 0.105$
$Y_{IgM} = -0.0023 \times ^{2} + 0.3532x + 55.3499$	0.8966	$P < 0.05$
DI		
$Y_{ACP} = -0.0377 \times ^{2} + 6.5724x + 78.4667$	0.9009	$P < 0.05$
$Y_{C3} = -0.0015 \times ^{2} + 0.2611x + 19.4513$	0.8547	$P = 0.055$
$Y_{C4} = -0.0004 \times ^{2} + 0.0634x + 5.7454$	0.8867	$P < 0.05$
$Y_{IgM} = -0.0022 \times ^{2} + 0.3467x + 50.8598$	0.8936	$P < 0.05$

[a] Values are means ± SD (n = 6), and different superscripts in the same row are significantly different ($P < 0.05$). LZ, Lysozyme activity (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).

3.4. Comparison of all indices between ferrous fumarate group and ferrous sulfate group

With dietary iron level up to 63.47 mg/kg, enteritis morbidity in **ferrous fumarate treatment** was not significantly different with **ferrous sulfate treatment** (**positive control, 73.50 mg iron/kg**).

In PI, with dietary iron level up to 35.38 mg/kg, content of IgM and mRNA levels of hepcidin, LEAP-2A, β-defensin-1, IL-12p40, IL-17D, TGF-β2, IL-4/13A, IL-4/13B, IL-10, IL-11, IκBα, IKKβ, TOR, S6K1, 4E-BP1 and 4E-BP2 in **ferrous fumarate treatment** were not significantly different with **ferrous sulfate treatment**. With dietary iron level up to 63.47 mg/kg, activities of LZ and ACP, contents of C3 and C4 and mRNA levels of LEAP-2B, Mucin2, IL-1β, IFN-γ, IL-8, IL-12p35, TGF-β1, IL-15, NF-κBp65 and IKKγ in **ferrous fumarate treatment** were not significantly different with **ferrous sulfate treatment**.

In MI, with dietary iron level up to 35.38 mg/kg, activity of ACP, content of IgM and mRNA levels of LEAP-2B, Mucin2, β-defensin-1, IFN-γ, IL-8, IL-12p35, IL-12p40, TGF-β2, IL-4/13A, IL-4/13B, IL-10, IL-11, IL-15, IκBα, IKKγ, TOR, S6K1 and 4E-BP2 in **ferrous fumarate treatment** were not significantly different with **ferrous sulfate treatment**. With dietary iron level up to 63.47 mg/kg, activity of LZ, content of C4 and mRNA levels of hepcidin, LEAP-2A, TNF-α, IL-1β, IL-17D, TGF-β1, NF-κBp65, IKKβ and 4E-BP1 in **ferrous fumarate treatment** were not significantly different with **ferrous sulfate treatment**. Besides, content of C3 in **ferrous fumarate treatment** was not significantly different with **ferrous sulfate treatment** with dietary iron level up to 86.43 mg/kg.

In PI, with dietary iron level up to 35.38 mg/kg, contents of C4 and IgM, and mRNA levels of LEAP-2B, IL-12p35, IL-12p40, IL-17D, TGF-β1, TGF-β2, IL-4/13A, IL-4/13B, IL-10, IKKβ, S6K1, 4E-BP1 and 4E-BP2 in **ferrous fumarate treatment** were not significantly different with **ferrous sulfate treatment**. With dietary iron level up to 63.47 mg/kg, activities of LZ and ACP, content of C3 and mRNA levels of hepcidin, LEAP-2A, Mucin2, β-defensin-1, TNF-α, IL-1β, IFN-γ, IL-8, IL-11, IL-15, NF-κBp65, IκBα, IKKγ and TOR in **ferrous fumarate treatment** were not significantly different with **ferrous sulfate treatment**.

4. Discussion

4.1. Iron deficiency decreased enteritis resistance of fish under infection of A. hydrophila

A. hydrophila is one of the most common pathogenic bacteria in freshwater fish, which causes severe enteritis, resulting in impaired immune function in intestine of fish [24,38]. Thus, we conducted a challenge test through *A. hydrophila* infection to investigate the

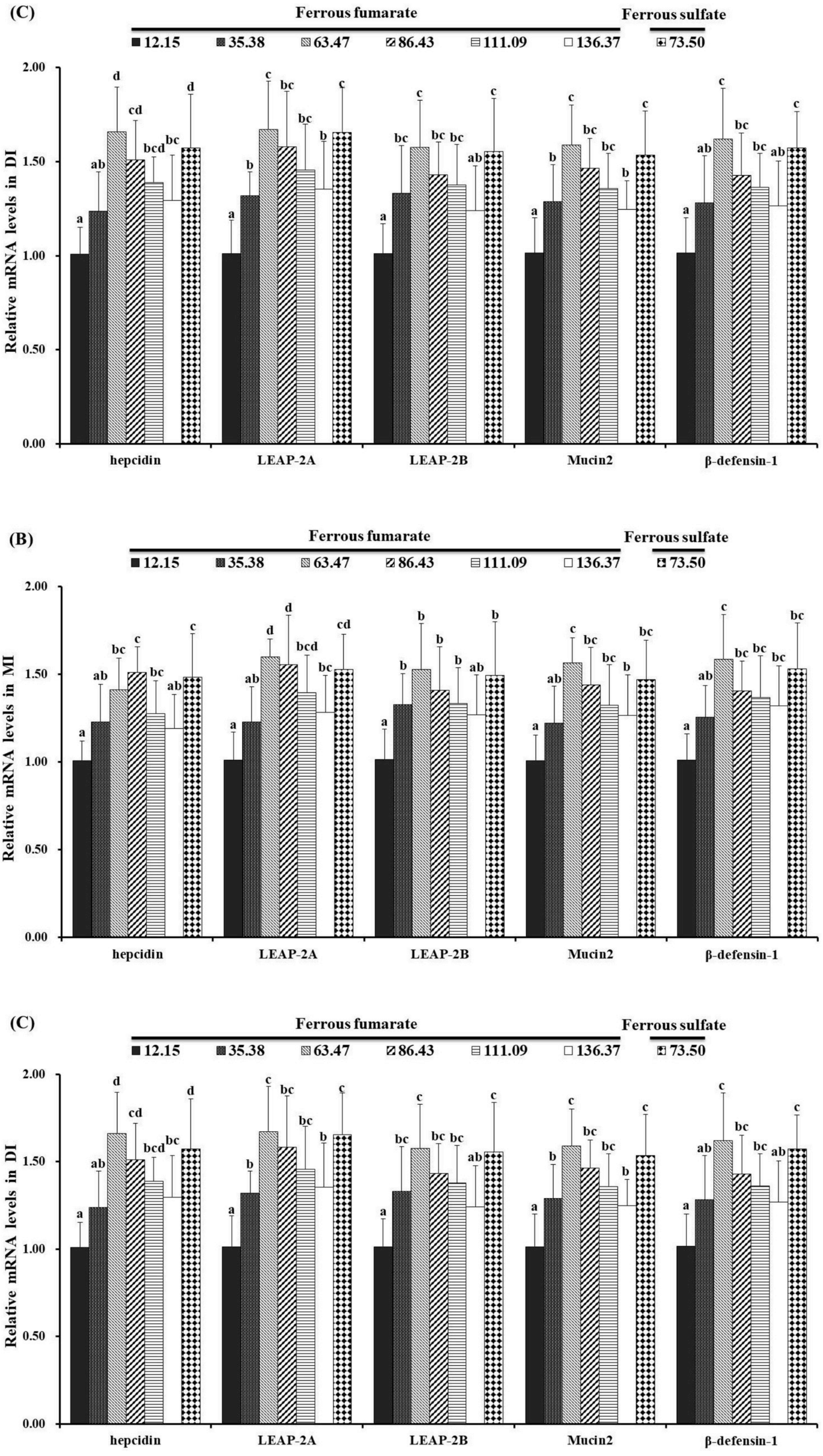

Fig. 3. Effects of dietary iron on anti-bacterial peptides in PI (A), MI (B) and DI (C) of on-growing grass carp (*Ctenopharyngodon idella*) under infection of *A. hydrophila*. Data represent means of six fish in each group. Values having different letters are significantly different ($P < 0.05$).

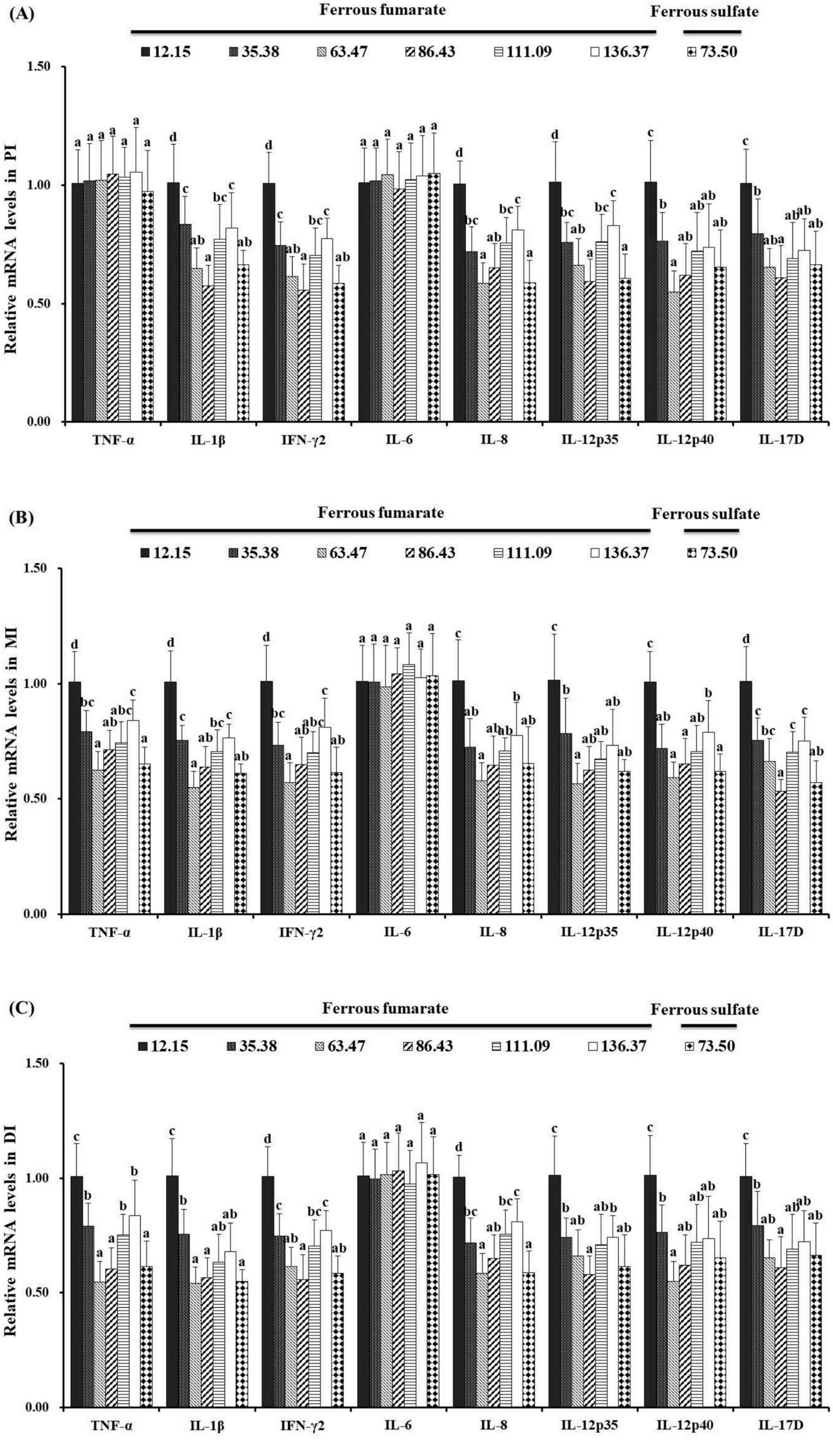

Fig. 4. Effects of dietary iron on pro-inflammatory cytokines in PI (A), MI (B) and DI (C) of on-growing grass carp (*Ctenopharyngodon idella*) under infection of *A. hydrophila*. Data represent means of six fish in each group. Values having different letters are significantly different ($P < 0.05$).

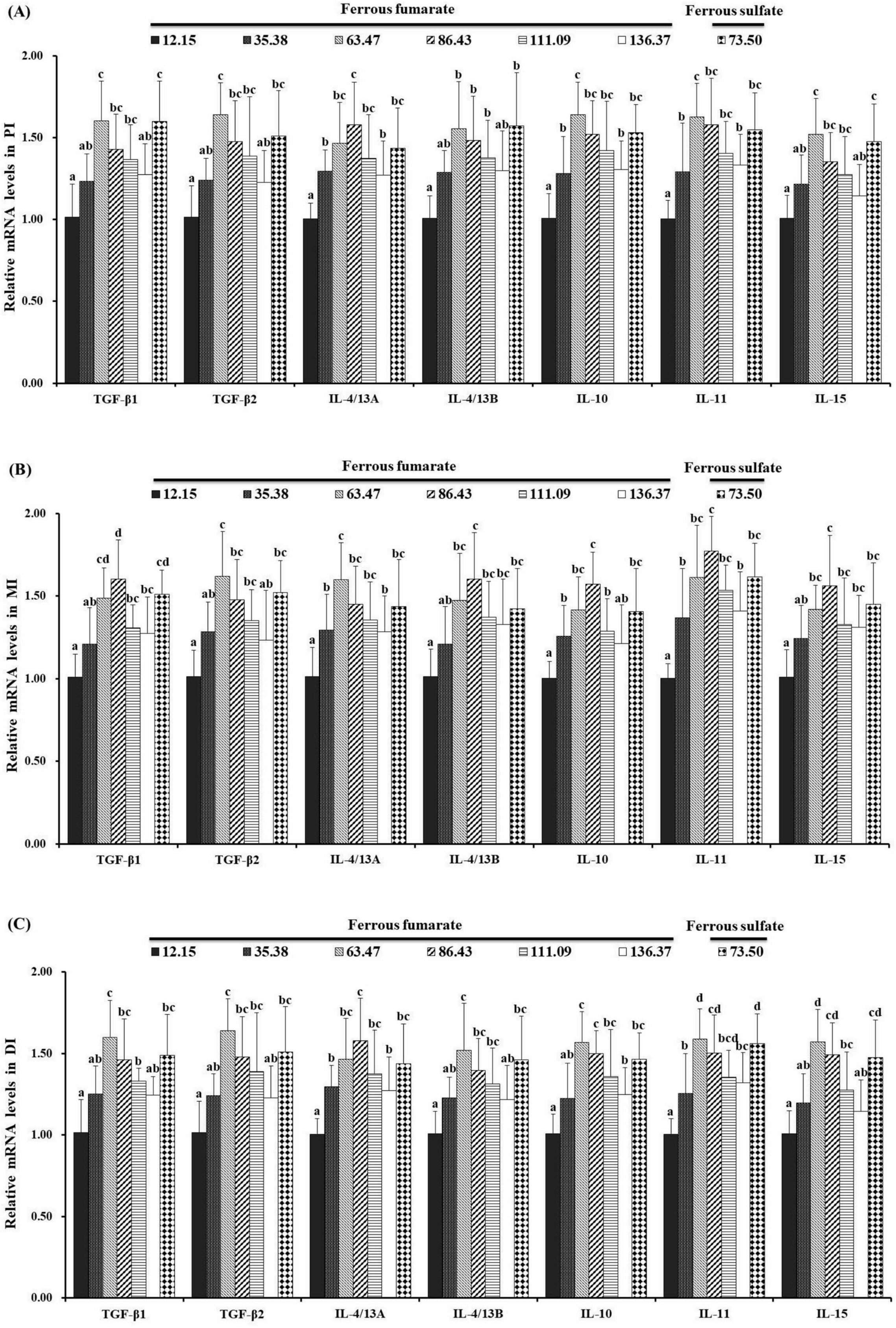

Fig. 5. Effects of dietary iron on anti-inflammatory cytokines in PI (A), MI (B) and DI (C) of on-growing grass carp (*Ctenopharyngodon idella*) under infection of *A. hydrophila*. Data represent means of six fish in each group. Values having different letters are significantly different ($P < 0.05$).

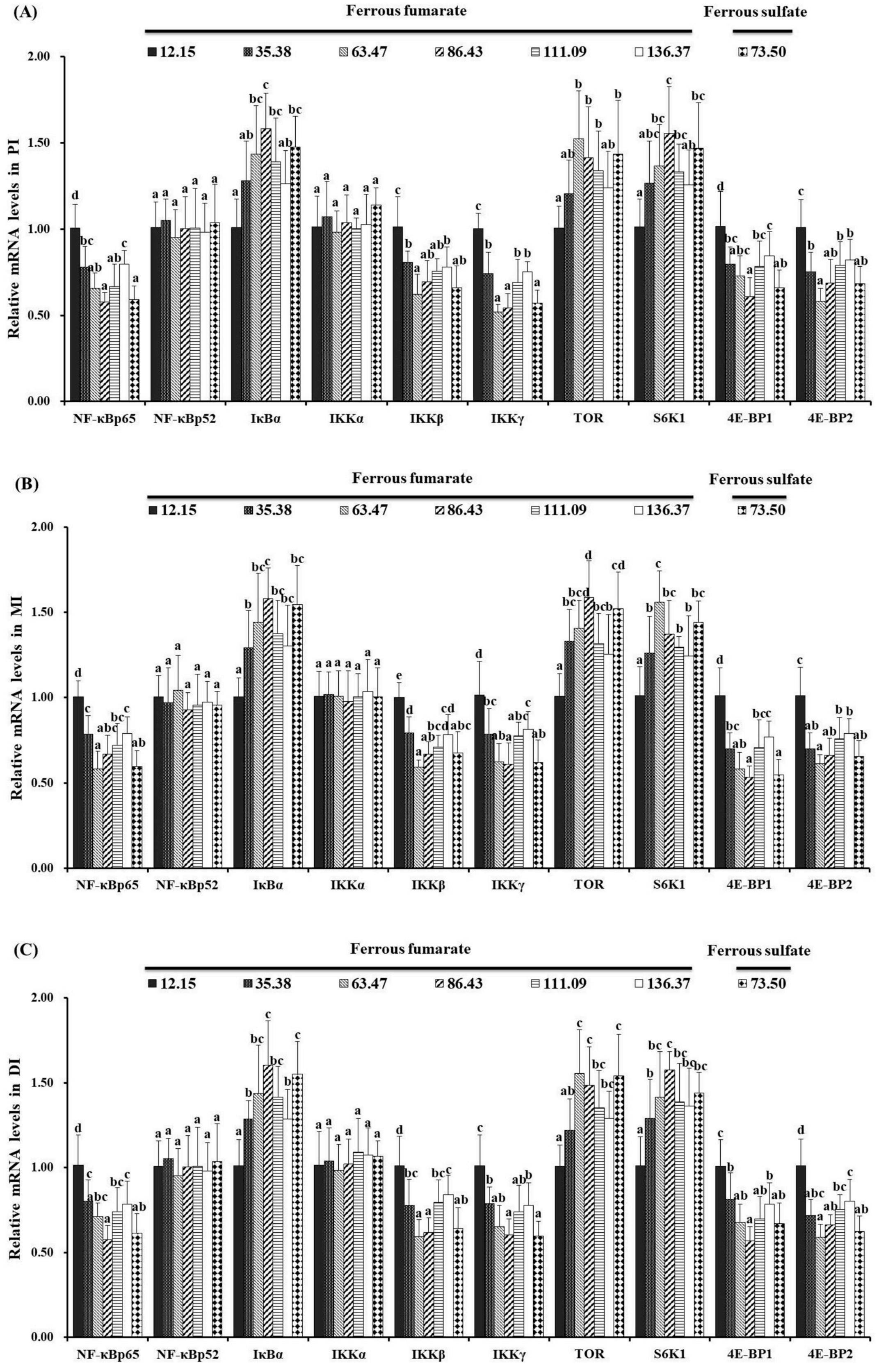

Fig. 6. Effects of dietary iron on inflammation related signaling molecules in PI (A), MI (B) and DI (C) of on-growing grass carp (*Ctenopharyngodon idella*) under infection of *A. hydrophila*. Data represent means of six fish in each group. Values having different letters are significantly different ($P < 0.05$).

influences of iron deficiency on enteritis of on-growing grass carp. In this study, iron deficiency increased the enteritis morbidity, indicating that iron deficiency could decrease the ability against *A. hydrophila* of fish.

4.2. Iron deficiency deceased antibacterial compounds in the intestine of fish

It has been reported that the ability defending against enteritis of fish is related to intestinal immune function [11,39]. In fish, humoral components including lysozyme, antibodies and complement factors, inhibited adherence and colonization of microorganisms in intestine, enhancing immune function [40]. Our data showed that iron deficiency decreased antibacterial compound concentrations and down-regulated antibacterial peptides mRNA levels in the intestine of on-growing grass carp, indicating that iron deficiency could impair intestinal immune function partially relating to the decreased antibacterial compounds of fish. These results were similar with our previous study [8,9].

4.3. Iron deficiency aggravated inflammation partially relating to NF-κB and TOR signaling in the intestine of fish

To our knowledge, intestinal immune function is also associated with inflammation which is partially related to cytokines in fish [41] and inflammation was driven by cytokines, including pro-inflammatory and anti-inflammatory cytokines, and up-regulated pro-inflammatory cytokines and down-regulated anti-inflammatory cytokines aggravated inflammation [42,43]. Our data showed that iron deficiency down-regulated anti-inflammatory cytokine mRNA levels, and up-regulated pro-inflammatory cytokine (except TNF-α in PI and IL-6 in three segments) mRNA levels in the intestine of on-growing grass carp, indicating that iron deficiency could impair intestinal immune function partially relating to aggravated inflammation of fish.

Surprisingly, our data showed that iron deficiency had no effects on the mRNA levels of TNF-α in PI, and IL-6 in three segments of on-growing grass carp. First, iron deficiency up-regulated the mRNA levels of TNF-α in MI and DI (except PI) of grass carp might be partially related to IGF-1. It was reported that in human, iron deficiency could decrease blood IGF-1 level [44]. Study revealed that IGF-1 could decrease mice aortic expression of TNF-α [45]. Besides, expression of IGF-1 in duodenum of chicken was much lower than that in jejunum and ileum [46]. Hence, we hypothesize that iron deficiency decreased IGF-1 level which was possibly higher expressed in MI and DI (rather than PI), resulting in up-regulated mRNA levels of TNF-α in MI and DI (rather than PI). But this hypothesis requires further investigation. Second, the unaffected mRNA levels of IL-6 in three segments of on-growing grass carp under iron deficiency might be partially related to progesterone. It was reported that in serum of does, iron deficiency could decrease progesterone concentration [47]. However, in peripheral blood mononuclear cells of human, progesterone had no effects on IL-6 expression [48]. Hence, we hypothesize that iron deficiency decreased the progesterone concentration, while progesterone had no effects on IL-6 expression in the intestine of fish, which requires further investigation.

Compared with our previous study [8,9], there are some differences, such as IFN-γ and IL-15. For the first, iron deficiency down-regulated mRNA levels of IFN-γ in the intestine rather than in head kidney, spleen, skin and gill of on-growing grass carp, which might be related to physiological characteristics of intestine. La Bonnardiere & Laude (1981) [49] have revealed that the intestine of pig with viral enteritis showed increased IFN production and the intestinal epithelial cell might play important roles in the synthesis of IFN. Besides, Chethan et al. (2017) [50] have also revealed that IFN level in piglets with rotavirus enteritis are significantly higher than those without enteritis. In this study, iron deficiency induced obvious enteritis in on-growing grass carp, which might explain the up-regulated mRNA level of IFN-γ in the intestine rather than head kidney, spleen, skin and gill of on-growing grass carp, but the underlying mechanisms requires further investigation. For the second, iron deficiency up-regulated mRNA levels of IL-15 in the intestine rather skin of on-growing grass carp might be related to erythropoietin (EPO). As stated in our previous study [9], the unaffected mRNA level of IL-15 in skin rather than head kidney and spleen might me related to EPO. Study revealed that EPO was not expressed in skin of human [51] but expressed in intestine of zebrafish (*Danio rerio*) [52]. Hence, we hypothesize that iron deficiency decreased the EPO level which was expressed in intestine (might not express in skin), causing down-regulated mRNA levels of IL-15 in the intestine (rather than skin) of fish.

Activation of the NF-κB proteins plays a central role in inflammation through the regulation of encoding pro-inflammatory cytokines transcription [53]. In fish, the activation of NF-κB relies on phosphorylation and then degradation of IκBα under the effect of IKK [54]. In this study, iron deficiency up-regulated the mRNA levels of NF-κBp65, IKKβ and IKKγ, and down-regulated the mRNA levels of IκBα in three segments of on-growing grass carp. Correlation analysis showed that pro-inflammatory cytokines (TNF-α only in MI and DI) mRNA levels were positively related to NF-κBp65 mRNA levels, and the mRNA levels of NF-κBp65 and IκBα were positively and negatively related to the mRNA levels of IKKβ and IKKγ (Table 4), respectively. All the data above suggested that iron deficiency up-regulated these pro-inflammatory cytokines (except TNF-α in MI and DI, and IL-6 in three segments) mRNA levels might be partially related to [(IKKβ, IKKγ)/IκBα/NF-κBp65] signaling. These results are similar with our previous study [8,9].

Interestingly, our data showed that iron deficiency had no effects on the mRNA levels of NF-κBp52 and IKKα in the intestine of on-growing grass carp. First, iron deficiency up-regulated the mRNA levels of NF-κBp65 (rather than NF-κBp52) in the intestine of on-growing grass carp might be partially related to the up-regulated IKKβ and unaffected IKKα mRNA levels, respectively. It has been reported that IKKβ could activate NF-κBp65, whereas IKKα could activate NF-κBp52 in human [55]. In this study, iron deficiency up-regulated the mRNA levels of IKKβ but had no effects on mRNA levels of IKKα in the intestine of on-growing grass carp, supporting our hypothesis. Second, iron deficiency up-regulated the mRNA levels of IKKβ and IKKγ (rather than IKKα) in the intestine of on-growing grass carp might be partially related to vitamin A. It has been reported that iron deficiency could decrease vitamin A content in liver of rat [56]. Previous study from our lab showed that vitamin A deficiency could up-regulate the mRNA levels of IKKβ and IKKγ (rather than IKKα) in intestine of on-growing grass carp [57]. Thus, we hypothesize that iron deficiency might decrease vitamin A content resulting to up-regulated mRNA levels of IKKβ and IKKγ (rather than IKKα) in the intestine of fish, which requires further investigation.

Noteworthily, in previous study, iron deficiency significantly up-regulated mRNA level of IKKα in gill of on-growing grass carp [8]. But in this study, iron deficiency had no effects on mRNA level of IKKα in the intestine of on-growing grass carp, which might be related to the lower ascorbic acid concentration in intestine. It has been reported that iron deficiency could decrease the blood ascorbic acid level in pig [58]. According to our previous study [29], low level of vitamin C significantly up-regulated mRNA level of IKKα in head kidney and spleen of on-growing grass carp. However, the ascorbate concentration in intestine of Nile tilapia (*Oreochromis niloticus* (L.)) is lower than that in gill [59]. According to the data above, we suppose that iron deficiency decreased the vitamin C level whose concentration might be higher in gill than that in the intestine, leading to the down-regulated mRNA levels of IKKα in gill rather than intestine of fish. However, the supposition needs further investigation.

In addition to the role in protein metabolism, mTOR with its downstream S6K1 and 4E-BP, and plays important roles in the regulation of anti-inflammatory cytokines in mammals [60]. In this study, iron deficiency down-regulated the TOR and S6K1 mRNA levels and up-regulate 4E-BP1 and 4E-BP2 mRNA levels in the intestine of on-growing

Table 4
Correlation analysis of parameters in the intestine of on-growing grass carp (*Ctenopharyngodon idella*)[a].

Independent parameters	Dependent parameters	PI		MI		DI	
		Correlation coefficients	P	Correlation coefficients	P	Correlation coefficients	*P*
NF-κBp65	TNF-α	–	–	+0.993	< 0.01	+0.887	< 0.05
	IFN-γ2	+0.984	< 0.01	+0.987	< 0.01	+0.979	< 0.01
	IL-1β	+0.960	< 0.01	+0.998	< 0.01	+0.930	< 0.01
	IL-8	+0.902	< 0.05	+0.987	< 0.01	+0.870	< 0.05
	IL-12p35	+0.966	< 0.01	+0.989	< 0.01	+0.985	< 0.01
	IL-12p40	+0.921	< 0.01	+0.981	< 0.01	+0.901	< 0.05
	IL-17D	+0.968	< 0.01	+0.896	< 0.05	+0.965	< 0.01
IKKβ	NF-κBp65	+0.917	< 0.05	+0.999	< 0.01	+0.903	< 0.05
	IκBα	−0.898	< 0.05	−0.920	< 0.01	−0.908	< 0.05
IKKγ	NF-κBp65	+0.953	< 0.01	+0.963	< 0.01	+0.981	< 0.01
	IκBα	−0.951	< 0.01	−0.967	< 0.01	−0.978	< 0.01
TOR	TGF-β1	+0.995	< 0.01	+0.948	< 0.01	+0.992	< 0.01
	TGF-β2	+0.993	< 0.01	+0.852	< 0.05	+0.993	< 0.01
	IL-4/13A	+0.922	< 0.01	+0.844	< 0.05	+0.922	< 0.01
	IL-4/13B	+0.989	< 0.01	+0.933	< 0.01	+0.996	< 0.01
	IL-10	+0.999	< 0.01	+0.987	< 0.01	+0.995	< 0.01
	IL-11	+0.988	< 0.01	+0.965	< 0.01	+0.990	< 0.01
	IL-15	+0.972	< 0.01	+0.967	< 0.01	+0.962	< 0.01
S6K1	TGF-β1	+0.799	= 0.057	+0.856	< 0.05	+0.836	< 0.05
	TGF-β2	+0.818	< 0.05	+0.987	< 0.01	+0.815	< 0.05
	IL-4/13A	+0.986	< 0.01	+0.994	< 0.01	+0.972	< 0.01
	IL-4/13B	+0.894	< 0.05	+0.835	< 0.05	+0.840	< 0.05
	IL-10	+0.863	< 0.05	+0.821	< 0.05	+0.887	< 0.05
	IL-11	+0.911	< 0.05	+0.850	< 0.05	+0.900	< 0.05
	IL-15	+0.771	= 0.073	+0.821	< 0.05	+0.810	= 0.051
4E-BP1	TGF-β1	−0.819	< 0.05	−0.945	< 0.01	−0.881	< 0.05
	TGF-β2	−0.843	< 0.05	−0.932	< 0.01	−0.875	< 0.05
	IL-4/13A	−0.990	< 0.01	−0.933	< 0.01	−0.991	< 0.01
	IL-4/13B	−0.910	< 0.05	−0.930	< 0.01	−0.886	< 0.05
	IL-10	−0.879	< 0.05	−0.967	< 0.01	−0.931	< 0.01
	IL-11	−0.923	< 0.01	−0.968	< 0.01	−0.923	< 0.01
	IL-15	−0.812	< 0.05	−0.955	< 0.01	−0.872	< 0.05
4E-BP2	TGF-β1	−0.952	< 0.01	−0.853	< 0.05	−0.951	< 0.01
	TGF-β2	−0.948	< 0.01	−0.934	< 0.01	−0.931	< 0.01
	IL-4/13A	−0.892	< 0.05	−0.947	< 0.01	−0.917	< 0.05
	IL-4/13B	−0.965	< 0.01	−0.832	< 0.05	−0.956	< 0.01
	IL-10	−0.956	< 0.01	−0.876	< 0.05	−0.933	< 0.01
	IL-11	−0.952	< 0.01	−0.889	< 0.05	−0.946	< 0.01
	IL-15	−0.963	< 0.01	−0.863	< 0.05	−0.907	< 0.05

[a] n = 6.

grass carp. Correlation analysis showed that mRNA levels of anti-inflammatory cytokines were positively related to the mRNA levels of TOR and S6K1, whereas negatively related to mRNA levels of 4E-BP1 and 4E-BP2 (Table 4). All the data above suggested that iron deficiency down-regulated mRNA levels of these anti-inflammatory cytokines might be partially related to [TOR/(S6K1, 4E-BP)] signaling in the intestine of fish. These results are similar wit hour previous study [8,9].

4.4. Iron excess impaired immune function in fish intestine

The present study showed that excess iron impaired intestinal immune function of on-growing grass carp under infection of *A. hydrophila*. The negative effects of excessive iron are still unclear, which might be explained by the following aspects. First, the decreased antibacterial capacity might be related to monocytes. It has been reported that iron excess could decrease phagocytic capacity of human monocytes, making it more susceptive to bacteria [61]. Second, the decreased antibacterial compounds by excessive iron might be partially related to phospholipids. In the previous study on rats, iron excess decreased the fluidity of membranes of antibacterial compound lysozyme and increased the lipid peroxidation of it, which destroyed the normal function of lysozyme [62]. Third, according to our previous study, iron excess increased oxidative damage in gill of on-growing grass carp [8]. The study on mice showed that oxidative damage could induce inflammation in serum [63]. All the data above indicated that the impaired intestinal immune function might be related to monocytes, phospholipids and oxidative damage, which requires investigation.

4.5. Effects of ferrous fumarate on intestinal immune function of on-growing grass carp, in contrast with ferrous sulfate

Our previous studies have proved better effects of ferrous fumarate on immune function in head kidney, spleen, skin and gill of on-growing grass carp compared with ferrous sulfate [8,9]. In this study, with dietary iron supplementation to 63.47 mg/kg in ferrous fumarate treatment, except C3 content in MI, other parameters were not significantly different or even better than ferrous sulfate treatment. These date indicated a more efficient effect of ferrous fumarate on intestinal immune function of on-growing grass carp. As for the possible reason, we have discussed in our previous study [8,9], which might be

Table 5
The optimal iron requirements based on different indices for on-growing grass carp (*Ctenopharyngodon idella*).

Indices	Regressive equation	R^2	*P*	Optimal dietary iron level
morbidity	$Y = 0.0039 \times ^{2} - 0.6503x + 42.0435$	0.8847	< 0.05	83.37 mg/kg
LZ activity in MI	$Y = -0.0128 \times ^{2} + 2.2198x + 102.1804$	0.6172	< 0.01	86.71 mg/kg
LZ activity in DI	$Y = -0.0253 \times ^{2} + 4.3208x + 90.8361$	0.7921	< 0.01	85.39 mg/kg

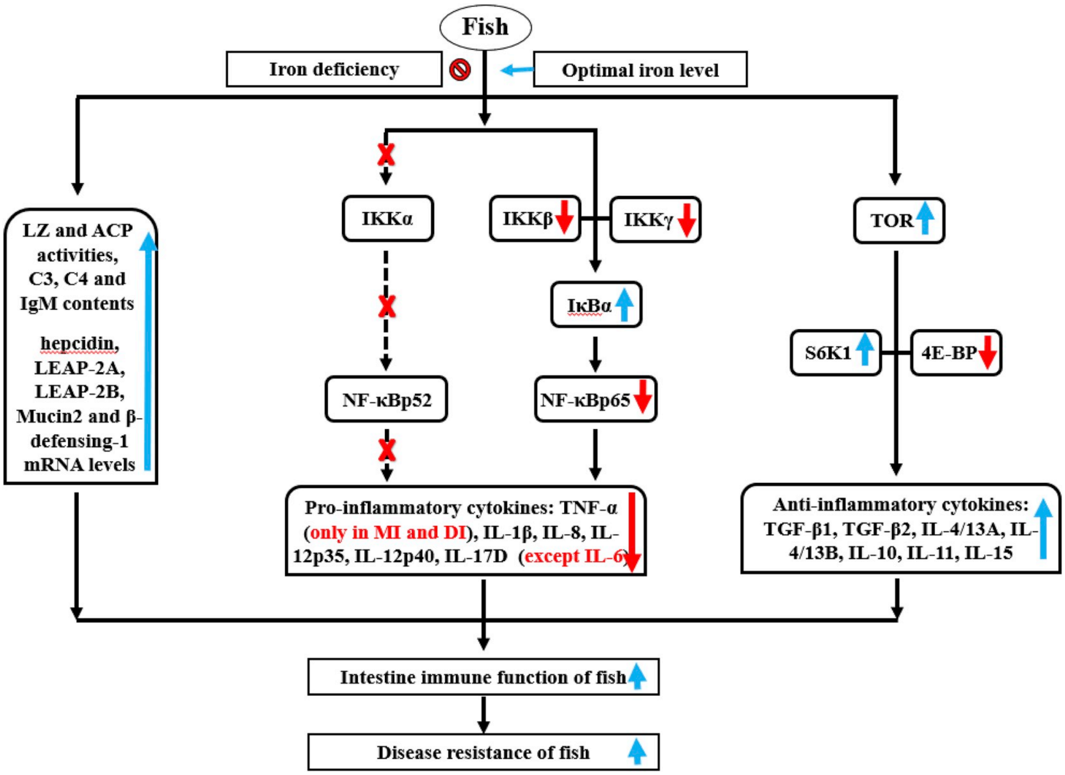

Fig. 7. The potential pathways about the effects of iron on intestinal immune function of fish.

correlated with the greater biological and stability of ferrous fumarate.

4.6. *Iron requirements based on different indices under the basis of ferrous fumarate*

According to the present study, iron deficiency increased enteritis morbidity and impaired the intestinal immune function of on-growing grass carp under infection of *A. hydrophila*. Hence, it is necessary to estimate the optimal iron requirements of on-growing grass carp. As stated above, *A. hydrophila* could lead to severe enteritis of fish [24], hence we estimated the optimal iron level based on the enteritis morbidity, which is 83.37 mg/kg. Besides, it has been reported that LZ is an important defense molecule in fish immune system [64]. Hence, because of the importance of LZ, we select LZ activity in MI and DI to estimate the optimal iron level of on-growing grass carp, which were 86.71 and 85.39 mg/kg, respectively. According to our previous study, the iron requirement based on growth performance is 75.65 mg/kg [9]. The requirements based on the ability against enteritis and LZ activity in MI and DI were slighter higher than that based on the growth performance, indicating that there may be a little higher requirement of iron for the enhancement of intestinal immune function of on-growing grass carp. (See Table 5)

5. Conclusions

In summary (Fig. 7), this study was for the first time to investigate the influences of iron deficiency on immune function in the intestine of fish. Compared with the optimal iron level, iron deficiency impaired disease resistance might be related to impaired immune function in intestine of fish, which is partially referred to the following two aspects. For the first, iron deficiency decreased antibacterial compounds concentrations, and down-regulated antibacterial peptides mRNA levels. For the second, iron deficiency aggravated inflammation referring to up-regulated pro-inflammatory cytokines mRNA levels (except TNF-α in PI and IL-6 in three segments) and down-regulated anti-inflammatory cytokines which were partially related to [IKKβ, IKKγ (rather than IKKα)/IκBα/NF-κBp65 (rather than NF-κBp52)] and [TOR/(S6K1, 4E-BP)] signaling, respectively. In addition, iron excess also impaired intestinal immune function of on-growing grass carp. Moreover, the effect of ferrous fumarate on intestinal immune function of on-growing grass carp is more efficient than ferrous sulfate. Finally, based on ability against enteritis as well as LZ activities in MI and DI, dietary iron requirements for on-growing grass carp (under the basis of ferrous fumarate) were estimated to be 83.37, 86.71 and 85.39 mg/kg diet, respectively.

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