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# Impaired intestinal immune barrier and physical barrier function by phosphorus deficiency: Regulation of TOR, NF-κB, MLCK, JNK and Nrf2 signalling in grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*

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ABSTRACT

In aquaculture, the occurrence of enteritis has increased and dietary nutrition is considered as one of the major strategies to solve this problem. In the present study, we assume that dietary phosphorus might enhance intestinal immune barrier and physical barrier function to reduce the occurrence of enteritis in fish. To test this assumption, a total of 540 grass carp (*Ctenopharyngodon idella*) were investigated by feeding graded levels of available phosphorus (0.95–8.75 g/kg diet) and then infection with *Aeromonas hydrophila*. The results firstly showed that phosphorus deficiency decreased the ability to combat enteritis, which might be related to the impairment of intestinal immune barrier and physical barrier function. Compared with optimal phosphorus level, phosphorus deficiency decreased fish intestinal antimicrobial substances activities or contents and down-regulated antimicrobial peptides mRNA levels leading to the impairment of intestinal immune response. Phosphorus deficiency down-regulated fish intestinal anti-inflammatory cytokines mRNA levels and up-regulated the mRNA levels of pro-inflammatory cytokines [except IL-1β and IL-12p35 in distal intestine (DI) and IL-12p40] causing aggravated of intestinal inflammatory responses, which might be related to the signalling molecules target of rapamycin and nuclear factor kappa B. In addition, phosphorus deficiency disturbed fish intestinal tight junction function and induced cell apoptosis as well as oxidative damage leading to impaired of fish intestinal physical barrier function, which might be partially associated with the signalling molecules myosin light chain kinase, c-Jun N-terminal protein kinase and NF-E2-related factor 2, respectively. Finally, based on the ability to combat enteritis, dietary available phosphorus requirement for grass carp (254.56–898.23 g) was estimated to be 4.68 g/kg diet.

## 1. Introduction

In fish, the intestinal immune barrier and physical barrier function is important for growth, which is a first line of defense against infection and plays a role in nutrient uptake [1,2]. Studies revealed that nutrients like manganese [3] and choline [4] could improve fish intestinal immune barrier and physical barrier function. Phosphorus is one of the major mineral nutrients for fish [5]. To our knowledge, phosphorus is involved in critical biological reactions, including protein phosphorylation, generation of high-energy carriers, and blood buffering [6]. It is also an important component of different cellular structures, including nucleic acids and phospholipid, which was necessary for pathogens and fish to maintain the normal homoeostatic control of the cell [7,8]. *Aeromonas hydrophila* is one of the most widespread pathogens in freshwater [9]. Rippey et al. reported that *A. hydrophila* growth closely depends on phosphorus [10]. With the consumption of phosphorus by *A. hydrophila*, fish might not have enough phosphorus to maintain normal biological reactions and cause a

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series of disease problems, such as enteritis and skin hemorrhage and lesions [9,11]. Fish must obtain phosphorus from their diets because of the low concentration and low absorption rate in natural waters [12,13]. Furthermore, it was reported that phosphorus is mainly absorbed in the proximal intestine of rainbow trout (*Oncorhynchus mykiss*), which closely relied on the function of type-IIb sodium-phosphate cotransporter [14]. However, whether phosphorus deficiency could impair the intestinal immune barrier and physical barrier function of fish needs further investigation.

Fish intestinal immune barrier function mainly depends on antimicrobial substances and cytokines [15]. It has been reported that the cytokines genes expressions were involved in target of rapamycin (TOR) and nuclear factor κB (NF-κB) signalling in fish [16,17]. However, no reports have conducted to investigate the effects of phosphorus on intestinal immune barrier of fish. Kanatani et al. found that a low level of phosphorus decreased insulin-like growth factor 1 (IGF-1) level in MC3T3-E1 cells [18]. The inhibition of IGF-1 induced the production of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in CD11b +cells [19]. Moreover, our previous study displayed that phosphorus deficiency increased reactive oxygen species (ROS) contents in grass carp [20]. It has been reported that ROS could inhibit mTOR activation in prostate cancer cells [21] and activate NF-κB in mouse embryonic fibroblasts [22]. These data indicate that there might be a relationship between phosphorus deficiency and antimicrobial substances, cytokines and its relevant signalling molecules in the intestine of fish, which is worth investigating.

Over and above intestinal immune barrier, fish intestinal physical barrier is another important guarantee of intestinal health, which is correlated with intercellular tight junction complexes (TJs) and epithelial cells [23]. A study showed that epithelial cells can be destructed by cell apoptosis and oxidative injury in rat [24]. Moreover, studies have revealed that the gene expression of TJs, cell apoptosis and antioxidant capacity could be regulated by myosin light chain kinase (MLCK) [25], c-Jun N-terminal protein kinase (JNK) [26] and NF-E2-related factor 2 (Nrf2) [27] in human, respectively. However, to date, the reports focused on the relationship between phosphorus and fish intestinal physical barrier are scarce. Feng et al. revealed that phosphorus deficiency decreased intestinal antioxidant enzymes such as superoxide dismutase (SOD) activities and then induced carp intestinal oxidative damage [28]. A study reported that phosphorus deficiency increased plasma cholesterol contents in black seabream (*Sparus macrocephalus*) [29]. High levels of cholesterol promoted MLCK expression in rabbits [30]. Meanwhile, it was proved that a low level of phosphorus decreased endothelial nitric oxide synthase (eNOS) level in rat endothelial cells [31]. The decline of eNOS could increase JNK activity and induce apoptosis in testes of adult rats [32]. In addition, phosphorus deficiency decreased NADPH oxidase activity in bovine aortic endothelial cells [33]. A low level of NADPH oxidase depressed the activation of Nrf2 in murine pulmonary epithelial cells [34]. Thus, phosphorus deficiency may affect TJs, apoptosis, antioxidant capacity and its relevant signalling molecules in the intestine of fish, which warrants further investigation.

The same growth trial was used in this study as our previous study [20], which is a part of a larger research effort to determine the effects of dietary phosphorus on fish growth and health status. Our previous study found that phosphorus deficiency decreased the intestinal digestion and absorption ability [35] and antioxidant capacity [28] in Jian carp. On the basic of those studies, the objectives of this study were intended to systematically and deeply investigate the influences of phosphorus on antimicrobial substances, cytokines, TJs, cell apoptosis and antioxidant capacity, as well as the relevant signalling molecules TOR, NF-κB, MLCK, JNK and Nrf2 in the intestine of fish. Those might provide a theoretical evidence to reveal the potential effects of phosphorus on fish intestinal health. Meanwhile, the phosphorus requirements for grass carp based on intestinal related indices were also evaluated, with a view to determining the optimal phosphorus requirements for aquaculture practices.

## 2. Materials and methods

### 2.1. Experimental diet and procedures

The formulation of the diets are the same as our previous study and showed in Table S1 [20]. Fish meal, casein, gelatin and rice gluten meal were used as dietary protein sources. Fish oil and soybean oil were used as dietary lipid sources. Six experimental diets were obtained by supplementing the basal diet with monosodium phosphate ($NaH_2PO_4{\cdot}2H_2O$, AR) which was from Chengdu Kelong Chemicals Reagents (Chengdu, China). The dietary total phosphorus were determined by the method of the AOAC (2000) [36], and final total phosphorus levels of the six experimental diets were 2.3 (un-supplemented), 4.0, 5.6, 7.6, 9.2 and 11.0 g/kg diet, respectively. According to digestibility trial in our previous study [20], the available phosphorus levels of six experimental diets were 0.95, 2.46, 3.96, 5.68, 7.10 and 8.75 g/kg diet, respectively. After being prepared completely, the diets were stored at −20 °C according to Xie et al. [35].

### 2.2. Growth trial

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University. After obtainment from fishery (Sichuan, China), grass carp were acclimated to experimental conditions for 4 weeks according to Xie et al. [35]. Subsequently, 540 fish with similar body weights (mean weight 256.22 ± 0.60 g) were randomly assigned to 18 experimental cages (1.4 L × 1.4 W × 1.4 H m), namely 30 fish per cage as described in our lab study [37]. Every cage was equipped with a disc of 100 cm diameter in the bottom to collect the uneaten feed according to Wu et al. [38]. Each experimental diet was randomly fed to fish one of triplicate cages of the six dietary treatments to apparent satiation, and fish were fed with respective diet four times daily according to Du et al. [39] for 60 days. The uneaten feed was collected 30 min after each meal as described by our previous study [40]. During the experiment, water temperature and pH were 27 ± 2 °C and 7.0 ± 0.4, respectively. The dissolved oxygen was higher than 6.0 mg/L. The treatment groups were under natural light and dark cycle as described by Wen et al. [41]. Before and during the experiment, daily water samples were collected and analyzed. Average waterborne phosphorus concentration was below 0.054 mg/L.

### 2.3. Challenge trial

The challenge test was conducted as described by our previous study [20]. *A. hydrophila* was friendly provided by College of Veterinary Medicine, Sichuan Agricultural University, China. After the growth trial, fifteen fish of similar body weight were obtained from each treatment group (3 small cages/group, N = 5 fish/small cage) according to Liu et al. [42] and moved to six new experimental cages, which were blocked in the middle, finally resulting in three independent parts equality with the same size as the original cages (1.4 length × 1.4 width × 1.4 height in metres). After 5 days acclimation according to Xu et al. [43], fish were intraperitoneally injected with 1.0 ml of $2.5 \times 10^9$ colony-forming units (cfu) $ml^{-1}$ *A. hydrophila* for each individual. The challenge dose was selected as an appropriate dose which could effectively induce inflammation and consequently enable the investigation on fish reactivity against a threatening disease without causing death according to our preliminary test (data was not shown). The challenge test was conducted for 14d according to Nya and Austin [44] and the managements were the same to the growth trial.

### 2.4. Sample collection and biochemical analysis

After the challenge trial, all fish from each treatment were anaesthetized with benzocaine according to Geraylou et al. [45]. After sacrificed, a scoring system was designed to evaluate the severity of fish enteritis with a semi-quantitative method according to Song et al. [9] and Kokou et al. [46], which was scored on a scale from 0 to 4 based on the percentage of intestinal mucosal lesions (from 0 to 100%). A score of increasing value represents higher (more severe) morphological alterations [9]. The calculation of enteritis morbidity was a weighted average score according to the methods of Bellows et al. [47] and Karter et al. [48], which were calculated from the following equation: weighted average (**Y**) = ($x_1$ $w_1$ + $x_2$ $w_2$… + $x_n$ $w_n$)/($w_1$ + $w_2$… + $w_n$), where x = the percentage of each score and w = score (0–4). Fish intestine was quickly removed on ice and divided into proximal intestine (PI), mid intestine (MI) and distal intestine (DI) according to the turning point described by Askarian et al. [49]. Then the intestinal samples were frozen in liquid nitrogen, then stored at −80 °C until analysis as described by Xu et al. [43].

Tissue homogenates of intestinal sample were prepared in 10 vol (w/v) of ice-cold normal saline and centrifuged at 6000 *g* for 20 min at 4 °C. The supernatant was conserved and used to determine the immune and antioxidant parameters, which was similar to Wu et al. [50]. The activities of LZ and acid phosphatase (ACP) were assayed according to Deng et al. [51]. The contents of C3 and C4 were measured with the method of Dawood et al. [52]. The contents of reactive oxygen species (ROS), malondialdehyde (MDA), protein carbonyl (PC) and glutathione (GSH) were assayed as described by Ni et al. [53]. The zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) activities were determined as described by Lambertucci et al. [54]. The catalase (CAT), glutathione-*S*-transferases (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were assayed according to Mieiro et al. [55].

### 2.5. Real-time polymerase chain reaction (PCR) analysis

Total RNA samples were isolated from the intestine using RNAiso Plus Kit (Takara, Dalian, China) according to the manufacturer's instructions, then quality and quantity were assessed using agarose gel (1%) electrophoresis and spectrophotometric (A260: 280 nm ratio) analysis, respectively, as described by Luo et al. [56]. Subsequently, RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa) according to the manufacturer's instructions. The specific primers for target and housekeeping genes and the annealing temperature for each gene are presented in Table S2. 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 normalize 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 to calculate the expression results after verifying that the primers amplified with an efficiency of approximately 100% according to Livak & Schmittgen [57].

### 2.6. Calculations and statistical analysis

All data were subjected to one-way analysis of variance followed by Duncan's multiple range tests to determine significant differences among treatment groups using the software SPSS 18.0 (SPSS Inc., Chicago, IL, USA) at a level of $P < .05$. The results are presented as the means ± SD. Heat map diagrams visualizing of phosphorus-changed gene expression were made using Excel 2013 (Microsoft Corporation) software. Broken-line analysis was used to evaluate the dietary phosphorus requirement of grass carp according to Xie et al. [35].

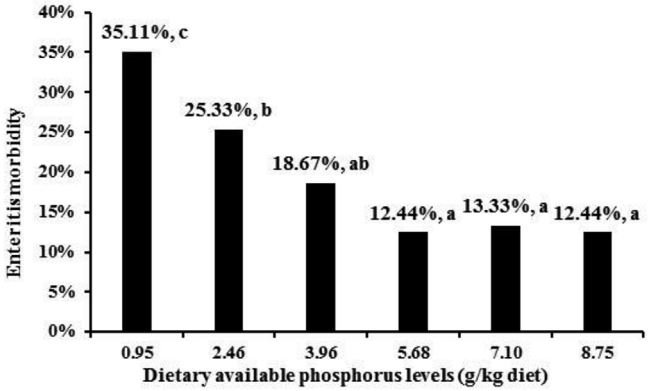


**Fig. 1. Effects of available phosphorus levels on enteritis morbidity in grass carp (*Ctenopharyngodon idella*).** Values having different letters are significantly different ($P < .05$).

## 3. Results

### 3.1. Enteritis and Enteritis morbidity of grass carp after infection with *A. hydrophila*

The effect of phosphorus on grass carp enteritis morbidity is showed in Fig. 1. The enteritis morbidity after infection with *A. hydrophila* gradually decreased as dietary available phosphorus levels increased to 3.96 g/kg diet in the intestine of grass carp, and then plateaued. Meanwhile, dietary phosphorus deficiency showed an obvious enteritis symptom (Fig. 2) after infection with *A. hydrophila* in grass carp that compared with optimal phosphorus level.

### 3.2. Immunological parameters in the intestine of grass carp after infection with *A. hydrophila*

#### 3.2.1. Antimicrobial substances activities or contents in the intestine

The effects of phosphorus on the activities or contents of intestinal antimicrobial substances of grass carp were presented in Table 1. The LZ activity as well as C3 and C4 contents in the PI of grass carp increased as the dietary available phosphorus levels increased to 5.68, 5.68 and 3.96 g/kg diet, respectively, and then plateaued. The LZ and ACP activities as well as C3 and C4 contents in the MI increased as the dietary available phosphorus levels increased to 5.68, 5.68, 3.96 and 3.96 g/kg diet, respectively, and plateaued thereafter. The LZ activity and C3 and C4 contents in the DI all increased as the dietary available phosphorus levels increased to 5.68 g/kg diet, and remained constant thereafter. The ACP activities were the highest for fish fed 3.96 and 5.68 g/kg available phosphorus diet in the PI and DI, respectively.

#### 3.2.2. Relative mRNA levels of antimicrobial peptides, cytokines and related signalling molecules in the intestine

The effects of dietary available phosphorus on immune-related indices in the PI, MI and DI of grass carp were presented in Fig. 3. In the PI, the mRNA levels of liver-expressed antimicrobial peptide 2A (LEAP-2A), LEAP-2B, β-defensin-1 and Mucin2 were up-regulated as the dietary available phosphorus levels increased to 2.46, 3.96, 2.46 and 3.96 g/kg diet, respectively, and then plateaued. In the MI, the gene expression of LEAP-2A, LEAP-2B, β-defensin-1 and Mucin2 were up-regulated as the dietary available phosphorus levels increased to 5.68, 3.96, 3.96 and 2.46 g/kg diet, respectively, and then plateaued. In the DI, as the dietary available phosphorus levels increased to 2.46, 3.96, 2.46 and 2.46 g/kg diet, respectively, the LEAP-2A, LEAP-2B, β-defensin-1 and Mucin2 mRNA levels were up-regulated and then plateaued. Dietary available phosphorus had no effect on the mRNA levels of hepcidin in the three intestinal segments of grass carp.

**In the PI**, the mRNA levels of IL-4/13A, IL-10, transforming growth

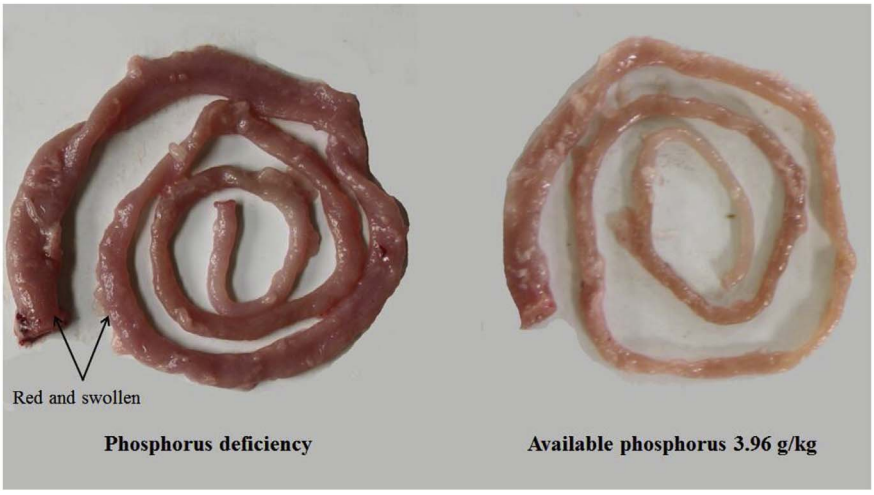


**Fig. 2.** Phosphorus deficiency led to an obvious enteritis symptom, compared to available phosphorus 3.96 g/kg diet, in grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*.

factor β1 (TGF-β1), inhibitor protein κBα (IκBα) and p70 S6 kinase (S6K1) were all gradually up-regulated as the dietary available phosphorus levels increased to 3.96 g/kg diet, simultaneously, IL-4/13B, IL-11 and TOR were significantly up-regulated as the dietary available phosphorus levels increased to 2.46, 5.68 and 2.46 g/kg diet ($P < .05$), and plateaued after. The gene expressions of IL-1β, IL-6, IL-15, NF-κB p52, 4E-BP1 and 4E-BP2 were down-regulated as the dietary available phosphorus levels increased to 3.96, 3.96, 5.68, 3.96, 3.96 and 3.96 g/kg diet, respectively, simultaneously, IL-8, IL-12p35, TNF-α, interferon γ2 (IFN-γ2), NF-κB p65, IκB kinases β (IKKβ) and IKKγ were significantly down-regulated as the dietary available phosphorus levels increased to 2.46, 3.96, 3.96, 5.68, 2.46, 2.46 and 3.96 g/kg diet ($P < .05$), respectively, and then kept constant. Fish fed 5.68 g/kg available phosphorus diet showed the highest TGF-β2 and the lowest IL-17D mRNA level. **In the MI**, IL-4/13A, IL-4/13B, IL-10, TGF-β1, TGF-β2, IκBα and TOR mRNA levels were gradually up-regulated as the dietary available phosphorus levels increased to 3.96, 3.96, 2.46, 3.96, 3.96, 2.46 and 3.96 g/kg diet, respectively, simultaneously, IL-11 and S6K1 mRNA levels were both significantly up-regulated as the dietary available phosphorus levels increased to 2.46 g/kg diet ($P < .05$), and plateaued thereafter. The mRNA levels of IL-15, IL-17D, NF-κB p52, IKKβ, IKKγ and 4E-BP2 were all gradually down-regulated as the dietary available phosphorus levels increased to 3.96 g/kg, simultaneously, IL-1β, IL-6, IL-12p35, TNF-α, IFN-γ2, NF-κB p65 and 4E-BP1 were all significantly down-regulated as the dietary available phosphorus levels increased to 2.46 g/kg diet ($P < .05$), and then plateaued. Fish fed 3.96 g/kg available phosphorus diet showed the lowest IL-8 mRNA level. **In the DI**, IL-4/13A, IL-10, IL-11, TGF-β1, TGF-β2,

**Table 1**
Effects of graded levels of available phosphorus on immune related parameters in the proximal intestine (PI), middle intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*).[a]

| | Available P in the diet (g/kg diet) | | | | | |
|---|---|---|---|---|---|---|
| | 0.95 | 2.46 | 3.96 | 5.68 | 7.10 | 8.75 |
| **LZ** | | | | | | |
| PI | 68.16 ± 4.53$^{a}$ | 131.80 ± 9.15$^{b}$ | 176.04 ± 31.24$^{c}$ | 205.20 ± 12.64$^{d}$ | 202.34 ± 13.64$^{d}$ | 197.58 ± 12.77$^{d}$ |
| MI | 111.50 ± 4.08$^{a}$ | 155.31 ± 7.20$^{b}$ | 186.24 ± 14.50$^{c}$ | 203.06 ± 13.95$^{d}$ | 206.91 ± 12.78$^{d}$ | 197.82 ± 12.65$^{cd}$ |
| DI | 134.39 ± 10.61$^{a}$ | 190.84 ± 14.31$^{b}$ | 224.21 ± 17.92$^{c}$ | 286.33 ± 15.18$^{d}$ | 287.89 ± 19.75$^{d}$ | 277.91 ± 14.22$^{d}$ |
| **ACP** | | | | | | |
| PI | 239.00 ± 20.97$^{a}$ | 315.75 ± 29.18$^{b}$ | 466.78 ± 35.80$^{e}$ | 427.77 ± 34.91$^{d}$ | 385.56 ± 33.07$^{c}$ | 359.33 ± 20.59$^{c}$ |
| MI | 253.70 ± 25.06$^{a}$ | 325.72 ± 28.19$^{b}$ | 413.65 ± 34.15$^{c}$ | 460.94 ± 41.21$^{d}$ | 449.28 ± 41.01$^{cd}$ | 454.09 ± 44.70$^{cd}$ |
| DI | 305.77 ± 21.47$^{a}$ | 367.89 ± 24.09$^{b}$ | 405.36 ± 36.59$^{bc}$ | 451.51 ± 44.07$^{d}$ | 415.56 ± 30.01$^{cd}$ | 370.08 ± 24.49$^{b}$ |
| **C3** | | | | | | |
| PI | 20.24 ± 1.61$^{a}$ | 24.43 ± 1.77$^{b}$ | 26.69 ± 2.66$^{bc}$ | 29.17 ± 2.76$^{cd}$ | 29.49 ± 1.98$^{d}$ | 28.44 ± 1.92$^{cd}$ |
| MI | 24.28 ± 2.00$^{a}$ | 28.87 ± 1.25$^{b}$ | 32.88 ± 2.71$^{c}$ | 34.17 ± 3.08$^{c}$ | 33.99 ± 3.03$^{c}$ | 33.65 ± 2.65$^{c}$ |
| DI | 26.03 ± 2.23$^{a}$ | 29.16 ± 1.03$^{a}$ | 33.49 ± 2.47$^{b}$ | 36.93 ± 2.89$^{c}$ | 37.39 ± 3.46$^{c}$ | 36.69 ± 3.50$^{bc}$ |
| **C4** | | | | | | |
| PI | 3.54 ± 0.29$^{a}$ | 5.80 ± 0.30$^{b}$ | 7.83 ± 0.64$^{c}$ | 7.40 ± 0.52$^{c}$ | 7.69 ± 0.76$^{c}$ | 7.65 ± 0.73$^{c}$ |
| MI | 6.36 ± 0.52$^{a}$ | 8.05 ± 0.72$^{b}$ | 9.94 ± 0.96$^{c}$ | 10.20 ± 0.79$^{c}$ | 10.70 ± 0.64$^{c}$ | 10.36 ± 0.95$^{c}$ |
| DI | 8.12 ± 0.80$^{a}$ | 10.47 ± 0.81$^{b}$ | 11.79 ± 1.12$^{c}$ | 13.88 ± 1.29$^{d}$ | 13.63 ± 1.31$^{d}$ | 13.31 ± 1.16$^{d}$ |
| **Regression** | | | | | | |
| $Y_{LZ\ in\ MI} = 19.3399x + 100.9299$ | | | | $Y_{max} = 202.59$ | $R^2 = 0.9499$ | $P < .05$ |
| $Y_{LZ\ in\ DI} = 31.2159x + 107.1016$ | | | | $Y_{max} = 284.04$ | $R^2 = 0.9918$ | $P < .01$ |
| $Y_{ACP\ in\ MI} = 45.0214x + 216.6208$ | | | | $Y_{max} = 454.77$ | $R^2 = 0.9797$ | $P = .01$ |
| $Y_{ACP\ in\ DI} = -6.1690x^2 + 69.0198x + 240.5729$ | | | | | $R^2 = 0.9574$ | $P < .01$ |
| $Y_{C3\ in\ PI} = 1.8439x + 19.1177$ | | | | $Y_{max} = 29.03$ | $R^2 = 0.9712$ | $P < .05$ |
| $Y_{C3\ in\ MI} = 2.8574x + 21.6578$ | | | | $Y_{max} = 33.67$ | $R^2 = 0.9987$ | $P < .05$ |
| $Y_{C3\ in\ DI} = 2.3569x + 23.7142$ | | | | $Y_{max} = 37.00$ | $R^2 = 0.9948$ | $P < .01$ |
| $Y_{C4\ in\ PI} = 1.4263x + 2.2175$ | | | | $Y_{max} = 7.64$ | $R^2 = 0.9992$ | $P < .05$ |
| $Y_{C4\ in\ MI} = 1.1900x + 5.1970$ | | | | $Y_{max} = 10.30$ | $R^2 = 0.9989$ | $P < .05$ |
| $Y_{C4\ in\ DI} = 1.1842x + 7.2018$ | | | | $Y_{max} = 13.61$ | $R^2 = 0.9897$ | $P < .01$ |

[a] Values are means ± SD (n = 6), and superscripted different letters in the same row are significantly different ($P < .05$). LZ: Lysozyme (U/mg protein); ACP: acid phosphatase (U/mg protein); C3: complement component 3 (mg/g protein); C4: complement component 4 (mg/g protein).

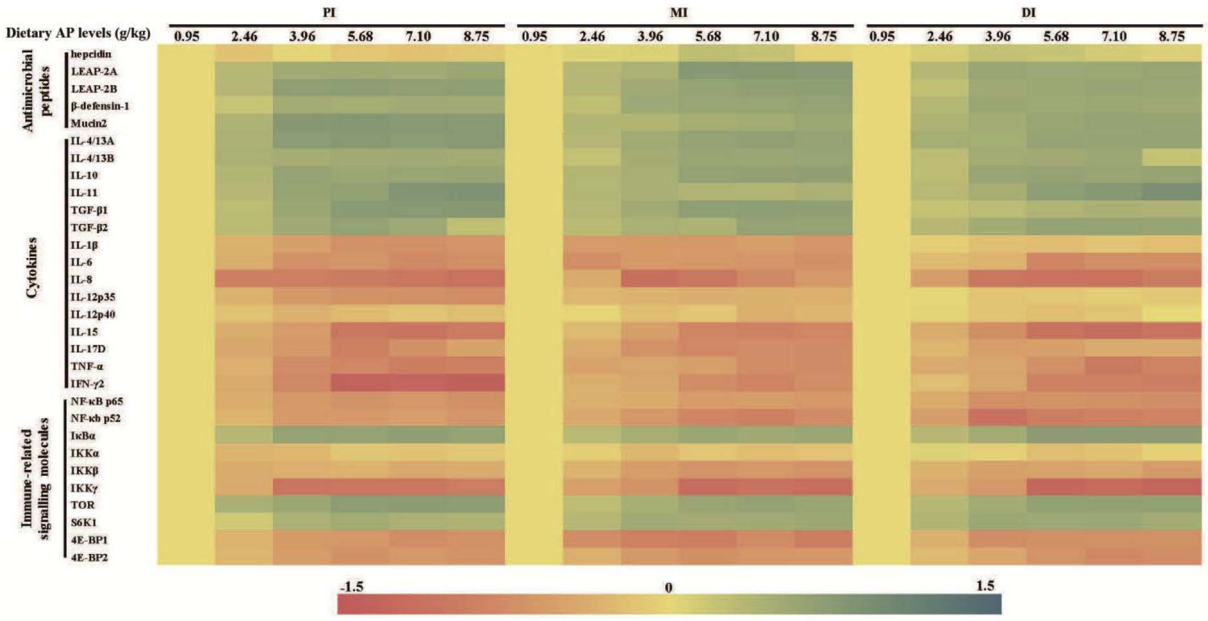


**Fig. 3.** Heat-map of phosphorus-changed expression of antimicrobial peptides, cytokines and related signalling molecules in the PI, MI and DI of grass carp after infected with *A. hydrophila*. The signal values of up-regulation (green) and down-regulation (red) were expressed as Log2Fold and ranged from 1.5 to −1.5 folds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

IκBα, TOR and S6K1 mRNA levels were up-regulated as the dietary available phosphorus levels increased to 2.46, 3.96, 5.68, 2.46, 2.46, 3.96, 3.96 and 2.46 g/kg diet, respectively, and then plateaued. Fish fed 7.10 g/kg available phosphorus diet had the highest IL-4/13B mRNA level. The gene expressions of IL-6, TNF-α, IFN-γ2 and 4E-BP2 were gradually down-regulated as the dietary available phosphorus levels increased to 5.68, 5.68, 5.68 and 3.96 g/kg diet, respectively, simultaneously, IL-8, IL-15, IL-17D, NF-κB p65, NF-κB p52, IKKβ, IKKγ and 4E-BP1 were significantly down-regulated as the dietary available phosphorus levels increased to 3.96, 5.68, 2.46, 3.96, 3.96, 2.46, 5.68 and 3.96 g/kg diet ($P < .05$), respectively, and then plateaued. Surprisingly, dietary phosphorus had no impact on the mRNA levels of IL-12p40 and IKKα in the three intestinal segments as well as IL-1β and IL-12p35 in the DI of grass carp.

### 3.3. Relative mRNA levels of TJs and MLCK in the intestine of grass carp

The effects of dietary available phosphorus on TJs and MLCK relative mRNA levels in the PI, MI and DI of grass carp were showed in Fig. 4. **In the PI**, the mRNA levels of ZO-1, ZO-2, claudin-b, -c, -f, -3c, -7a, -7b and −11 were gradually up-regulated as the dietary available phosphorus levels increased to 3.96, 2.46, 3.96, 3.96, 2.46, 2.46, 3.96, 3.96 and 5.68 g/kg diet, respectively, and then plateaued. **In the MI**, ZO-1, ZO-2, claudin-b, -c and -f mRNA levels were gradually down-regulated as the dietary available phosphorus levels increased to 3.96, 3.96, 3.96, 2.46 and 3.96 g/kg diet, respectively, simultaneously, claudin-3c, -7b and −11 were all significantly up-regulated as the dietary available phosphorus levels increased to 2.46 g/kg diet ($P < .05$), and then reminded constant. **In the DI**, the gene expressions of ZO-1, ZO-2, occludin, claudin-b, -c, -f and -3c were gradually up-regulated as the dietary available phosphorus levels increased to 3.96, 2.46, 3.96, 3.96, 2.46, 2.46 and 3.96 g/kg diet, respectively, simultaneously, claudin-7b and −11 were both significantly up-regulated as the dietary available phosphorus levels increased to 2.46 g/kg diet ($P < .05$), and then plateaued. Compared with dietary phosphorus deficiency, phosphorus supplementation down-regulated the mRNA levels of claudin-12 and MLCK in the three intestinal segments. Fish fed 5.68 and 3.96 g/kg available phosphorus diet showed the highest mRNA levels of claudin-7a in the MI and DI, respectively. Interestingly, phosphorus deficiency had no influence on claudin-15a and claudin-15b mRNA levels in the intestine and occludin mRNA levels in the PI and MI of grass carp.

### 3.4. Relative mRNA levels of apoptosis-related parameters in the intestine of grass carp

As shown in Fig. 4, **In the PI**, the apoptotic protease activating factor-1 (Apaf-1) and Bcl-2 associated X protein (Bax) mRNA levels were both gradually down-regulated as the dietary available phosphorus levels increased to 3.96 g/kg diet, respectively, simultaneously, the cysteinyl aspartic acid-protease 3 (caspase-3), −7, −8, −9 and JNK were significantly down-regulated as the dietary available phosphorus levels increased to 5.68, 3.96, 3.96, 3.96 and 2.46 g/kg diet ($P < .05$), respectively, and plateaued after. As the dietary available phosphorus levels increased to 2.46, 3.96 and 3.96 g/kg diet, the mRNA levels of B-cell lymphoma protein-2 (Bcl-2), myeloid cell leukemia-1 (Mcl-1) and inhibitor of apoptosis proteins (IAP) were up-regulated, respectively, and then plateaued. **In the MI**, the mRNA levels of caspase-3, -8, Apaf-1 and JNK were gradually down-regulated as the dietary available phosphorus levels increased to 5.68, 3.96, 5.68 and 3.96 g/kg diet, respectively, simultaneously, caspase-7, -9 and Bax were all significantly down-regulated as the dietary available phosphors increased to 2.46 g/kg diet ($P < .05$), and then plateaued. As the dietary available phosphorus levels increased to 3.96, 3.96 and 2.46 g/kg diet, Bcl-2, Mcl-1 and IAP mRNA levels were gradually up-regulated, respectively, and reminded constant thereafter. **In the DI**, caspase-3, -9, Bax and JNK mRNA levels were gradually down-regulated as the dietary available phosphorus levels increased to 5.68, 3.96, 2.46 and 5.68 g/kg diet, respectively, simultaneously, caspase-7 and Apaf-1 were both significantly down-regulated as the dietary available phosphorus levels increased to 2.46 g/kg diet ($P < .05$), and then plateaued. As the dietary available phosphorus levels increased to 3.96 g/kg diet, Bcl-2, Mcl-1 and IAP gene expressions were all gradually up-regulated, and then plateaued. Fish fed 5.68 g/kg available phosphorus diet had the lowest mRNA levels of caspase-8 and FasL. However, dietary phosphorus had no impact on FasL mRNA levels in the PI and MI of grass carp.

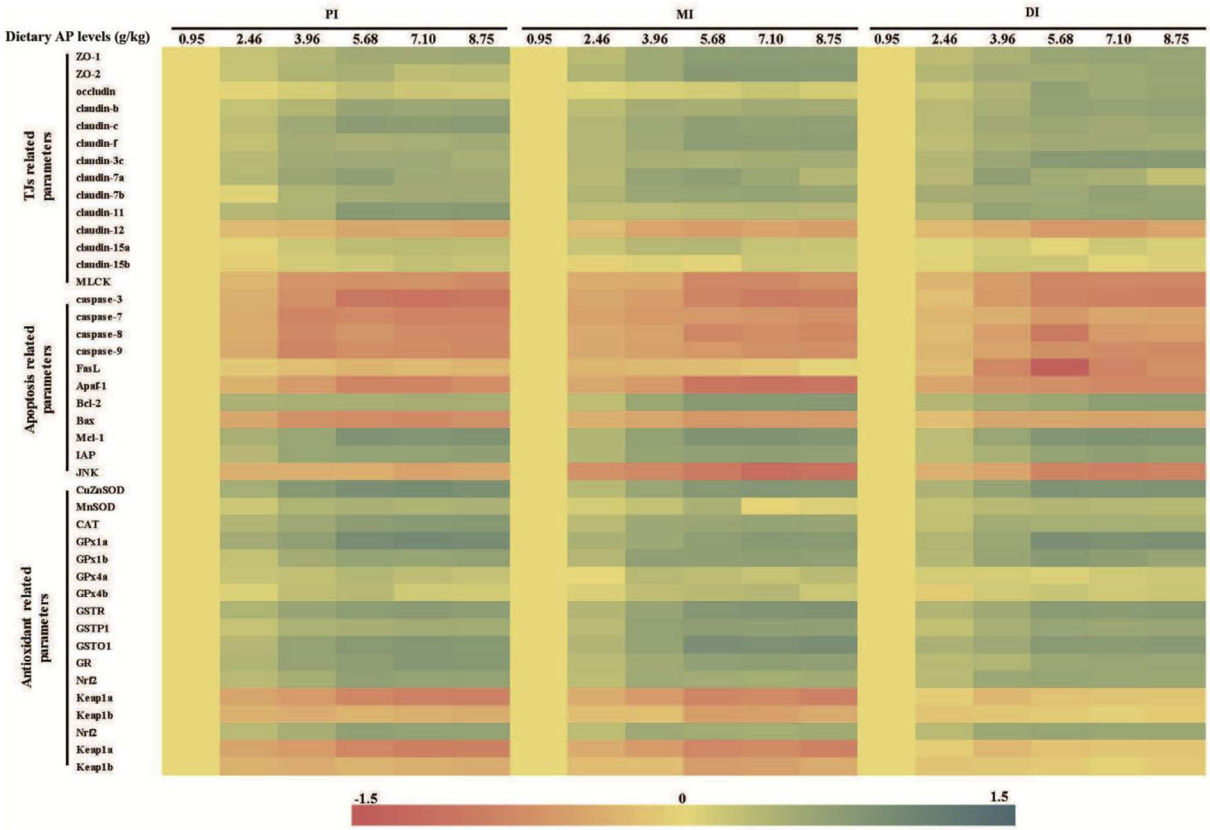


**Fig. 4.** Heat-map of phosphorus-changed expression of TJs, apoptosis and antioxidant-related parameters in the PI, MI and DI of grass carp after infected with *A. hydrophila*. The signal values of up-regulation (green) and down-regulation (red) were expressed as Log2Fold and ranged from 1.5 to −1.5 folds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### *3.5. The activities, contents and relative mRNA levels of antioxidant-related parameters in the intestine of grass carp*

Intestinal ROS, MDA and PC contents and antioxidant-related parameters of grass carp were presented in Table 2. **In the PI**, the contents of ROS, MDA and PC all gradually decreased as the dietary available phosphorus levels increased to 5.68 g/kg diet, and MDA and PC contents plateaued after, but an increase was showed in ROS content. The CuZnSOD, CAT, GPx and GST activities increased as the dietary available phosphorus levels increased to 5.68, 3.96, 3.96 and 5.68 g/kg diet, respectively, and then plateaued. The MnSOD and GR activities and GSH content increased as the dietary available phosphorus levels increased to 5.68, 3.96 and 3.96 g/kg diet, respectively, and then decreased. **In the MI**, the contents of ROS, MDA and PC all gradually decreased as the dietary available phosphorus levels increased to 5.68 g/kg diet, and MDA and PC contents plateaued after, but an increase was showed in ROS content. The CuZnSOD, MnSOD, CAT, GPx and GST activities increased as the dietary available phosphorus levels increased to 3.96, 2.46, 3.96, 5.68 and 5.68 g/kg diet, where the response reach a plateau. Fish fed 3.96 g/kg available phosphorus diet showed the maximum GR activity and GSH content. **In the DI**, the ROS, MDA and PC contents decreased as the dietary available phosphorus levels increased to 5.68, 5.68 and 3.96 g/kg diet, and there was no significant change in MDA content thereafter, but increases were observed in ROS and PC contents. The CuZnSOD, MnSOD, CAT, GPx, GST and GR activities and GSH content increased as the dietary available phosphorus levels increased to 3.96, 3.96, 3.96, 3.96, 5.68, 3.96 and 3.96 g/kg diet, respectively, and then plateaued.

As shown in Fig. 4, **in the PI**, the mRNA levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GSTR, GSTP1, GSTO1, GR and Nrf2 were gradually up-regulated as the dietary available phosphorus levels increased to 3.96, 2.46, 3.96, 5.68, 3.96, 3.96, 2.46, 3.96, 3.96 and 3.96 g/kg diet, respectively, and then plateaued. The Keap1a and Keap1b mRNA levels were down-regulated as the dietary available phosphorus levels increased to 3.96 and 2.46 g/kg diet, respectively, and plateaued thereafter. **In the MI**, the gene expressions of CuZnSOD, CAT, GPx1a, GPx1b, GSTR, GSTP1, GSTO1, GR and Nrf2 were gradually up-regulated as the dietary available phosphorus levels increased to 3.96, 3.96, 3.96, 3.96, 3.96, 3.96, 5.68, 3.96 and 3.96 g/kg diet, respectively, and then plateaued. Fish fed 5.68 g/kg available phosphorus diet showed the highest mRNA level of MnSOD. Compared with dietary phosphorus deficiency, phosphorus supplementation down-regulated the Keap1a and Keap1b mRNA levels in the PI and MI. **In the DI**, as the dietary available phosphorus levels increased to 3.96, 2.46, 2.46, 5.68, 3.96, 3.96, 3.96, 3.96, 2.46 and 2.46 g/kg diet, the mRNA levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GSTR, GSTP1, GSTO1, GR and Nrf2 were gradually up-regulated, respectively, and then plateaued. Dietary phosphorus had no effect on GPx4a and GPx4b mRNA levels in the three intestinal segments and Keap 1 mRNA level in the DI of grass carp.

## 4. Discussion

Our previous study had shown that dietary available phosphorus requirement for the optimal growth of grass carp was determined to be 4.10 g/kg diet [20], and this study used the same animal trial (the final mean weight and SD of Groups 1–6 are 611 ± 12$^{a}$, 734 ± 22$^{b}$, 881 ± 17$^{c}$, 885 ± 16$^{c}$, 898 ± 18$^{c}$ and 889 ± 9$^{c}$ g, respectively). Fish growth is partly relied on the intestinal health which is associated with intestinal immune barrier and physical barrier function [43]. Thus, the present study, for the first time, explores the effects of phosphorus on the intestinal immune barrier and physical barrier function of fish and the related signalling pathways.

**Table 2**
Effects of graded levels of available phosphorus on antioxidant related parameters in the proximal intestine (PI), middle intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*)[a].

| | Available P in the diet (g/kg diet) | | | | | |
|---|---|---|---|---|---|---|
| | 0.95 | 2.46 | 3.96 | 5.68 | 7.10 | 8.75 |
| **PI** | | | | | | |
| ROS | 100.00 ± 8.14$^{e}$ | 76.78 ± 5.46$^{d}$ | 57.63 ± 4.57$^{b}$ | 45.31 ± 1.71$^{a}$ | 58.27 ± 2.95$^{b}$ | 68.51 ± 6.64$^{c}$ |
| MDA | 33.44 ± 2.79c | 27.62 ± 2.72b | 25.48 ± 2.48b | 18.97 ± 1.46a | 19.23 ± 1.66a | 19.73 ± 1.88a |
| PC | 7.59 ± 0.69$^{d}$ | 5.76 ± 0.47$^{c}$ | 4.07 ± 0.33$^{b}$ | 3.17 ± 0.23$^{a}$ | 3.03 ± 0.30$^{a}$ | 3.32 ± 0.29$^{a}$ |
| CuZnSOD | 9.65 ± 0.76$^{a}$ | 10.94 ± 1.08$^{b}$ | 11.87 ± 0.95$^{bc}$ | 13.11 ± 0.81$^{cd}$ | 13.34 ± 1.30$^{d}$ | 13.01 ± 1.13$^{cd}$ |
| MnSOD | 8.03 ± 0.70$^{a}$ | 8.47 ± 0.59$^{ab}$ | 8.54 ± 0.79$^{ab}$ | 9.96 ± 0.96$^{c}$ | 9.37 ± 0.55$^{bc}$ | 9.04 ± 0.54$^{b}$ |
| CAT | 1.90 ± 0.06$^{a}$ | 2.06 ± 0.18$^{ab}$ | 2.19 ± 0.15$^{bc}$ | 2.35 ± 0.12$^{c}$ | 2.26 ± 0.22$^{bc}$ | 2.34 ± 0.22$^{c}$ |
| GPx | 109.19 ± 9.38$^{a}$ | 118.43 ± 11.21$^{ab}$ | 125.40 ± 10.29$^{bc}$ | 137.07 ± 13.62$^{c}$ | 139.64 ± 13.73$^{c}$ | 136.71 ± 8.68$^{c}$ |
| GST | 56.72 ± 4.95$^{a}$ | 69.59 ± 4.60$^{b}$ | 78.67 ± 1.90$^{c}$ | 88.22 ± 5.59$^{d}$ | 86.09 ± 7.51$^{d}$ | 88.14 ± 6.49$^{d}$ |
| GR | 41.93 ± 2.74$^{a}$ | 54.71 ± 4.21$^{bc}$ | 63.61 ± 3.48$^{d}$ | 56.39 ± 4.45$^{c}$ | 55.53 ± 3.57$^{bc}$ | 51.57 ± 3.41$^{b}$ |
| GSH | 4.07 ± 0.36$^{a}$ | 6.05 ± 0.52$^{b}$ | 8.20 ± 0.53$^{d}$ | 7.32 ± 0.63$^{c}$ | 7.08 ± 0.64$^{c}$ | 6.87 ± 0.51$^{c}$ |
| **MI** | | | | | | |
| ROS | 100.00 ± 5.68$^{e}$ | 79.17 ± 7.79$^{d}$ | 59.50 ± 5.35$^{b}$ | 52.02 ± 4.82$^{a}$ | 57.10 ± 3.96$^{ab}$ | 69.30 ± 5.77$^{c}$ |
| MDA | 27.63 ± 2.63$^{c}$ | 25.62 ± 1.17$^{c}$ | 21.57 ± 1.95$^{b}$ | 15.85 ± 1.54$^{a}$ | 17.17 ± 1.50$^{a}$ | 17.66 ± 1.66$^{a}$ |
| PC | 3.59 ± 0.33$^{d}$ | 3.00 ± 0.28$^{c}$ | 2.78 ± 0.24$^{bc}$ | 2.58 ± 0.22$^{ab}$ | 2.32 ± 0.23$^{a}$ | 2.35 ± 0.21$^{a}$ |
| CuZnSOD | 7.21 ± 0.64$^{a}$ | 9.35 ± 0.50$^{b}$ | 10.43 ± 0.82$^{c}$ | 10.61 ± 0.76$^{c}$ | 10.72 ± 0.82$^{c}$ | 10.55 ± 0.31$^{c}$ |
| MnSOD | 5.09 ± 0.48$^{a}$ | 5.57 ± 0.37$^{ab}$ | 6.07 ± 0.40$^{b}$ | 6.07 ± 0.49$^{b}$ | 5.98 ± 0.56$^{b}$ | 5.71 ± 0.56$^{b}$ |
| CAT | 1.93 ± 0.07$^{a}$ | 2.05 ± 0.06$^{ab}$ | 2.17 ± 0.12$^{bc}$ | 2.22 ± 0.11$^{c}$ | 2.29 ± 0.13$^{c}$ | 2.22 ± 0.18$^{c}$ |
| GPx | 117.26 ± 11.15$^{a}$ | 132.75 ± 12.36$^{b}$ | 146.01 ± 12.70$^{bc}$ | 160.69 ± 14.99$^{cd}$ | 159.69 ± 10.92$^{cd}$ | 162.90 ± 15.54$^{d}$ |
| GST | 60.25 ± 2.80$^{a}$ | 74.49 ± 4.13$^{b}$ | 83.82 ± 3.07$^{c}$ | 91.81 ± 3.52$^{d}$ | 90.98 ± 4.39$^{d}$ | 92.66 ± 3.21$^{d}$ |
| GR | 41.03 ± 2.91$^{a}$ | 45.03 ± 2.82$^{ab}$ | 49.93 ± 4.70$^{c}$ | 45.63 ± 2.47$^{bc}$ | 46.62 ± 4.41$^{bc}$ | 42.94 ± 3.28$^{ab}$ |
| GSH | 4.14 ± 0.34$^{a}$ | 6.36 ± 0.48$^{b}$ | 7.93 ± 0.74$^{d}$ | 8.00 ± 0.67$^{d}$ | 7.23 ± 0.49$^{c}$ | 6.58 ± 0.56$^{bc}$ |
| **DI** | | | | | | |
| ROS | 100.00 ± 5.20$^{d}$ | 69.65 ± 6.39$^{c}$ | 64.14 ± 5.08$^{bc}$ | 51.78 ± 5.09$^{a}$ | 55.40 ± 4.59$^{a}$ | 63.25 ± 3.23$^{b}$ |
| MDA | 32.93 ± 3.25$^{c}$ | 27.73 ± 2.73$^{b}$ | 25.54 ± 1.85$^{b}$ | 22.21 ± 2.03$^{a}$ | 24.79 ± 2.42$^{ab}$ | 25.87 ± 2.04$^{b}$ |
| PC | 3.35 ± 0.28$^{d}$ | 2.86 ± 0.27$^{bc}$ | 2.29 ± 0.21$^{a}$ | 2.62 ± 0.22$^{b}$ | 3.01 ± 0.18$^{c}$ | 3.11 ± 0.31$^{cd}$ |
| CuZnSOD | 8.77 ± 0.79$^{a}$ | 9.80 ± 0.93$^{a}$ | 11.01 ± 1.06$^{b}$ | 11.31 ± 0.98$^{b}$ | 11.20 ± 1.07$^{b}$ | 11.21 ± 0.71$^{b}$ |
| MnSOD | 6.92 ± 0.44$^{a}$ | 7.98 ± 0.49$^{b}$ | 9.45 ± 0.90$^{c}$ | 9.28 ± 0.70$^{c}$ | 9.47 ± 0.87$^{c}$ | 9.20 ± 0.89$^{c}$ |
| CAT | 2.35 ± 0.18$^{a}$ | 2.55 ± 0.25$^{a}$ | 2.81 ± 0.18$^{b}$ | 2.83 ± 0.13$^{b}$ | 2.87 ± 0.24$^{b}$ | 2.88 ± 0.15$^{b}$ |
| GPx | 96.84 ± 4.17$^{a}$ | 108.59 ± 4.72$^{b}$ | 125.78 ± 11.61$^{c}$ | 123.80 ± 9.38$^{c}$ | 121.07 ± 11.43$^{c}$ | 123.65 ± 10.36$^{c}$ |
| GST | 74.62 ± 6.41$^{a}$ | 88.24 ± 4.11$^{b}$ | 95.47 ± 4.27$^{c}$ | 105.65 ± 8.02$^{d}$ | 102.59 ± 5.08$^{d}$ | 104.93 ± 6.34$^{d}$ |
| GR | 44.05 ± 3.10$^{a}$ | 55.28 ± 2.42$^{b}$ | 60.86 ± 5.75$^{bc}$ | 64.49 ± 5.15$^{c}$ | 62.40 ± 3.55$^{bc}$ | 63.17 ± 3.24$^{c}$ |
| GSH | 4.88 ± 0.29$^{a}$ | 5.99 ± 0.58$^{b}$ | 7.91 ± 0.55$^{c}$ | 7.93 ± 0.79$^{c}$ | 8.10 ± 0.76$^{c}$ | 7.90 ± 0.36$^{c}$ |
| **Regression** | | | | | | |
| $Y_{PC\ in\ PI} = -0.9483x + 8.2405$ | | | | $Y_{min} = 3.17$ | $R^2 = 0.9688$ | $P < .05$ |
| $Y_{CuZnSOD\ in\ PI} = 0.7214x + 9.0388$ | | | | $Y_{max} = 13.15$ | $R^2 = 0.9964$ | $P < .01$ |
| $Y_{MnSOD\ in\ PI} = -0.0488x^2 + 0.6415x + 7.2907$ | | | | | $R^2 = 0.7178$ | $P = .150$ |
| $Y_{CAT\ in\ PI} = 0.0945x + 1.8169$ | | | | $Y_{max} = 2.28$ | $R^2 = 0.9954$ | $P < .05$ |
| $Y_{GPx\ in\ PI} = 5.3851x + 104.4415$ | | | | $Y_{max} = 137.81$ | $R^2 = 0.9938$ | $P = .050$ |
| $Y_{GR\ in\ PI} = = -0.9010x^2 + 9.4871x + 35.5685$ | | | | | $R^2 = 0.8024$ | $P = .088$ |
| $Y_{GSH\ in\ PI} = -0.1508x^2 + 1.7518x + 2.7309$ | | | | | $R^2 = 0.8556$ | $P = .055$ |
| $Y_{ROS\ in\ MI} = 1.9621x^2 - 23.1209x + 121.4036$ | | | | | $R^2 = 0.9917$ | $P < .01$ |
| $Y_{MDA\ in\ MI} = -2.5214x + 30.8933$ | | | | $Y_{min} = 16.90$ | $R^2 = 0.9683$ | $P < .05$ |
| $Y_{PC\ in\ MI} = -0.2060x + 3.6630$ | | | | $Y_{min} = 2.41$ | $R^2 = 0.9103$ | $P < .05$ |
| $Y_{CuZnSOD\ in\ MI} = 1.0700x + 6.3652$ | | | | $Y_{max} = 10.58$ | $R^2 = 0.9658$ | $P = .118$ |
| $Y_{GST\ in\ MI} = 6.5983x + 56.0672$ | | | | $Y_{max} = 91.81$ | $R^2 = 0.9732$ | $P < .05$ |
| $Y_{ROS\ in\ DI} = 1.6864x^2 - 20.6730x + 115.8293$ | | | | | $R^2 = 0.9641$ | $P < .01$ |
| $Y_{MDA\ in\ DI} = 0.3734x^2 - 4.4813x + 36.7755$ | | | | | $R^2 = 0.9551$ | $P = .01$ |
| $Y_{CuZnSOD\ in\ DI} = 0.7427x + 8.0330$ | | | | $Y_{max} = 11.18$ | $R^2 = 0.9977$ | $P < .05$ |
| $Y_{MnSOD\ in\ DI} = 0.8420x + 6.0458$ | | | | $Y_{max} = 9.35$ | $R^2 = 0.9908$ | $P = .061$ |
| $Y_{CAT\ in\ DI} = 0.1520x + 2.1987$ | | | | $Y_{max} = 2.85$ | $R^2 = 0.9955$ | $P < .05$ |
| $Y_{GPx\ in\ DI} = 9.6122x + 86.7868$ | | | | $Y_{max} = 123.58$ | $R^2 = 0.9880$ | $P = .070$ |
| $Y_{GSH\ in\ DI} = 1.0076x + 3.7873$ | | | | $Y_{max} = 7.96$ | $R^2 = 0.9764$ | $P = .098$ |

[a] Values are means ± SD (n = 6), and superscripted different letters in the same row are significantly different ($P < .05$). ROS, reactive oxygen species (% DCF florescence); MDA, malondialdehyde (nmol/g tissue); PC, protein carbonyl (nmol/mg protein); CuZnSOD, copper/zinc superoxide dismutase (U/mg protein); MnSOD, manganese superoxide dismutase (U/mg protein); CAT, catalase (U/mg protein); GPx, glutathione peroxidase (U/mg protein); GST, glutathione-S-transferase (U/mg protein); GR, glutathione reductase (U/g protein); GSH, glutathione (mg/g protein).

### *4.1. Phosphorus availability and phosphorus deficiency increased enteritis morbidity in fish after infection with A. hydrophila*

Phosphorus is an essential component of fish diets and rich in plant seeds, bone and mineral. Plant seeds contain most of their phosphorus in the form of phytic acid, which cannot be hydrolysed in the fish intestine due to negligible activity of phytase in the mucosa [58]. Phosphorus exists primarily in bone as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$)] with a low utilization for fish. The digestibility of inorganic phosphate in mineral is affected by their solubility [59]. In general, availability of phosphorus is dependent on dietary source, with inorganic and animal phosphorus sources being more available to fish than plant feedstuff sources [60]. Thus, a critical point is the determination of phosphorus availability in fish. According to our previous study, the available phosphorus contents in the six experimental diets were estimated to be 0.95 (un-supplemented control), 2.46, 3.96, 5.68, 7.10 and 8.75 g/kg diet, respectively.

*A. hydrophila* is one of the most widespread pathogens in freshwater [9]. When fish infected with pathogenic bacterium, like *A. hydrophila*, bacterium would attach to the intestinal tract and grow fast enough to

prevent them from being flushed out by the movement of food through the digestive tract [61]. This process requires large amounts of phosphorus and its metabolites, such as nucleic acids, phospholipid, ATP and so on [8,10]. It was reported that plasma phosphorus contents decreased in laying hens after bacterial infection [62]. Studies showed that a low level of phosphorus decreased fish disease resistance [63,64], which might be partly related to NF-κB signalling pathway [65]. Moreover, with the multiplication of *A. hydrophila* in fish intestine, an imbalanced microbiota disturbed these immune regulatory functions and contribute to the development of intestine diseases [66]. Thus, after fish intestine infection with *A. hydrophila*, fish might not have enough phosphorus to maintain normal biological reactions and cause an imbalance of intestinal microbiota. As a result of this, a high mortality of enteritis is induced by *A. hydrophila* in fish [67,68]. Therefore, after the feeding trial, we conducted a challenge test by infection fish with *A. hydrophila* to investigate the influences of phosphorus on the ability of fish to combat enteritis through Song et al. establishing enteritis model in grass carp [9]. In this study, phosphorus deficiency increased enteritis morbidity, indicating that phosphorus deficiency decreased the ability of fish to combat enteritis. In addition, fish intestinal health is also closely related to intestinal immune barrier and physical barrier function [43]. Therefore, we next investigated the effects of phosphorus on the intestinal immune barrier and physical barrier function of grass carp.

### *4.2. Phosphorus deficiency impaired intestinal immune barrier function partly relating to NF-κB and TOR signalling in fish*

#### *4.2.1. Phosphorus deficiency decreased intestinal immune response of fish*

In fish, immune response is an essential part of immune function, which closely depends on LZ, ACP, complement factors and antimicrobial peptides [69,70]. Thus, our study for the first time found that, compared with optimal level, phosphorus deficiency decreased LZ and ACP activities, C3 and C4 contents as well as down-regulated antimicrobial peptides including LEAP-2A, LEAP-2B, β-defensin-1 and Mucin2 mRNA levels in the three intestinal segments of grass carp, suggesting that phosphorus deficiency decreased intestinal immune response of fish. Interestingly, phosphorus deficiency had no effects on hepcidin expressions in the three intestinal segments of grass carp. This interesting phenomenon might be related to IL-10. Lee et al. reported that IL-10 had no effect on hepcidin expressions in murine primary hepatocytes [71]. In this study, phosphorus deficiency down-regulated IL-10 mRNA levels in the three intestinal segments of grass carp, supporting our hypothesis. In addition, the immune function is also correlated with inflammatory responses, which could be regulated by cytokines in fish [15]. Thus, this study next to explore the effects of phosphorus on the intestinal cytokines of grass carp as well as its potential mechanism.

#### *4.2.2. Phosphorus deficiency aggravated intestinal inflammatory responses partly relating to TOR and NF-κB signalling in fish*

Studies demonstrated that down-regulation of anti-inflammatory cytokines such as IL-10 and up-regulation of pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α could aggravate the inflammatory response in fish [72,73]. In this study, compared with optimal phosphorus level, phosphorus deficiency down-regulated anti-inflammatory cytokines IL-4/13A, IL-4/13B, IL-10, IL-11, TGF-β1 and TGF-β2 mRNA levels and up-regulated pro-inflammatory cytokines IL-6, IL-8, IL-15, IL-17D, TNF-α and IFN-γ2 mRNA levels in the three intestinal segments as well as IL-1β and IL-12p35 in the PI and MI of grass carp, indicating that phosphorus deficiency aggravated intestinal inflammatory responses partly by controlling the gene expressions of cytokines in fish.

Surprisingly, our data revealed that phosphorus had no effects on the mRNA levels of IL-1β and IL-12p35 in the DI as well as IL-12p40 in the three intestinal segments of grass carp. First, phosphorus deficiency up-regulated IL-1β mRNA levels in the PI and MI, but had no effect on IL-1β mRNA level in the DI, which might be partly related to the absorption of cholesterol in different intestinal segments. It was reported that phosphorus deficiency increased plasma cholesterol content in black seabream [29], and cholesterol absorption primarily occurred in the proximal and mid small intestine of mice [74,75]. A report revealed that the high level of cholesterol up-regulated IL-1β mRNA level in brain of mice [76]. Hence, we suppose that phosphorus deficiency might increase the absorption of cholesterol leading to up-regulated of IL-1β mRNA levels in the PI and MI (rather than DI) of fish. However, this supposition needs further characterization. Second, we found that phosphorus deficiency up-regulated the mRNA levels of IL-12p35 in the PI and MI, but had no effects on IL-12p35 in the DI and IL-12p40 in the three intestinal segments. This interesting phenomenon is same to our previous study in the head kidney and spleen [20], which might be correlated with the different mRNA levels of IL-1β in the three intestinal segments of grass carp. A study reported that IL-1β could up-regulate the IL-12p35 (rather than IL-12p40) mRNA level in the mature DCs [77]. As above mentioned, in this study, phosphorus deficiency up-regulated IL-1β mRNA levels in the PI and MI, but had no effect on IL-1β mRNA level in the DI. A further correlation analysis showed that IL-12p35 mRNA levels were positively related to IL-1β mRNA levels in the PI and MI of grass carp (Table 3), supporting our hypothesis.

Early evidence has shown that inhibition of mTOR/(S6K1, 4E-BP) signalling pathway decreased production of anti-inflammatory cytokines IL-10 in human mature DCs [78]. In the present study, compared with optimal phosphorus level, phosphorus deficiency down-regulated the mRNA levels of TOR and S6K1, and up-regulated the mRNA levels of 4E-BP1 and 4E-BP2 in the three intestinal segments of grass carp. Correlation analysis showed that the anti-inflammatory cytokines IL-4/13A, IL-4/13B, IL-10, IL-11, TGF-β1 and TGF-β2 mRNA levels were positively correlated with TOR mRNA levels in the three intestinal segments of grass carp (Table 3). These results suggested that phosphorus deficiency down-regulated anti-inflammatory cytokines gene expressions partly referring to the TOR/(S6K1, 4E-BP) signalling pathway in the three intestinal segments of fish.

It has been reported that the signalling IKK/IκBα/NF-κB is responsible for the genes transcription of pro-inflammatory cytokines in mammalian [79], and activated NF-κB p65 could up-regulate pro-inflammatory cytokines IL-1β and TNF-α mRNA levels in fish [17]. In the study, compared with optimal phosphorus level, phosphorus deficiency up-regulated the mRNA levels of NF-κB p65, NF-κB p52, IKKβ and IKKγ and down-regulated IκBα mRNA levels in the three intestinal segments of grass carp. A further correlation analysis presented that the mRNA levels of pro-inflammatory cytokines IL-1β (not in DI), IL-6, IL-8, IL-12p35 (not in DI), IL-15, IL-17D, TNF-α and IFN-γ were positively related to NF-κB p65 and NF-κB p52 in the three intestinal segments of grass carp (Table 3). These data indicated that the up-regulation of those pro-inflammatory cytokines mRNA levels by phosphorus deficiency might be partially related to (IKKβ, IKKγ)/IκBα/(NF-κB p65, NF-κB p52) signalling in the three intestinal segments of fish.

Interestingly, phosphorus deficiency up-regulated IKKβ and IKKγ (but not IKKα) mRNA levels in the three intestinal segments of grass carp, which may be concerned with phospholipids. In the intestine of grass carp, dietary phospholipids un-supplemented group up-regulated IKKβ, IKKγ (but not IKKα) mRNA levels [80]. Phosphorus deficiency could reduce the synthesis of phospholipid in mammalian cells [81]. According to above studies, we hypothesize that phosphorus deficiency might decrease phospholipids contents causing up-regulated of IKKβ, IKKγ (but not IKKα) mRNA levels in the intestine of fish, a further investigation is required for this hypothesis.

#### *4.2.3. Brief summary: phosphorus deficiency impaired intestinal immune barrier function in different intestinal segments of grass carp*

As stated above, our study first of all demonstrated that phosphorus deficiency impaired intestinal immune barrier function in the three intestinal segments of grass carp, as is showed in following two aspects:

**Table 3**
Correlation coefficient of parameters in the intestine.

| Independent parameters | Dependent parameters | PI | | MI | | DI | |
|---|---|---|---|---|---|---|---|
| | | Correlation coefficients | *P* | Correlation coefficients | *P* | Correlation coefficients | *P* |
| NF-κB p65 | IL-1β | +0.980 | < .01 | +0.927 | < .01 | – | – |
| | IL-6 | +0.987 | < .01 | +0.895 | < .05 | +0.854 | < .05 |
| | IL-8 | +0.954 | < .01 | +0.794 | = .059 | +0.987 | < .01 |
| | IL-12p35 | +0.991 | < .01 | +0.956 | < .01 | – | – |
| | IL-15 | +0.942 | < .01 | +0.953 | < .01 | +0.950 | < .01 |
| | IL-17D | +0.920 | < .01 | +0.966 | < .01 | +0.950 | < .01 |
| | TNF-α | +0.979 | < .01 | +0.957 | < .01 | +0.911 | < .05 |
| | IFN-γ2 | +0.953 | < .01 | +0.979 | < .01 | +0.894 | < .05 |
| NF-κB p52 | IL-1β | +0.970 | < .01 | +0.900 | < .05 | – | – |
| | IL-6 | +0.982 | < .01 | +0.862 | < .05 | +0.759 | = .080 |
| | IL-8 | +0.930 | < .01 | +0.876 | = .022 | +0.981 | < .01 |
| | IL-12p35 | +0.994 | < .01 | +0.955 | < .01 | – | – |
| | IL-15 | +0.935 | < .01 | +0.978 | < .01 | +0.893 | < .05 |
| | IL-17D | +0.899 | = .015 | +0.985 | < .01 | +0.980 | < .01 |
| | TNF-α | +0.981 | < .01 | +0.936 | < .01 | +0.836 | < .05 |
| | IFN-γ2 | +0.953 | < .01 | +0.988 | < .01 | +0.802 | = .055 |
| IL-1β | IL-12p35 | +0.987 | < .01 | +0.986 | < .01 | – | – |
| IκBα | NF-κB p65 | −0.982 | < .01 | −0.977 | < .01 | −0.921 | < .01 |
| | NF-κB p52 | −0.984 | < .01 | −0.980 | < .01 | −0.842 | < .05 |
| | IKKβ | −0.816 | < .05 | −0.996 | < .01 | −0.871 | < .05 |
| | IKKγ | −0.996 | < .01 | −0.987 | < .01 | −0.998 | < .01 |
| TOR | IL-4/13A | +0.963 | < .01 | +0.997 | < .01 | +0.962 | < .01 |
| | IL-4/13B | +0.950 | < .01 | +0.983 | < .01 | +0.746 | = .089 |
| | IL-10 | +0.948 | < .01 | +0.985 | < .05 | +0.969 | < .01 |
| | IL-11 | +0.955 | < .01 | +0.839 | < .01 | +0.946 | < .01 |
| | TGF-β1 | +0.977 | < .01 | +0.997 | < .01 | +0.996 | < .01 |
| | TGF-β2 | +0.767 | = .075 | +0.945 | < .01 | +0.996 | < .01 |
| MLCK | ZO-1 | −0.955 | < .01 | −0.970 | < .01 | −0.996 | < .01 |
| | ZO-2 | −0.877 | < .05 | −0.992 | < .01 | −0.958 | < .01 |
| | occludin | – | – | – | – | −0.978 | < .01 |
| | claudin-b | −0.939 | < .01 | −0.918 | < .01 | −0.996 | < .01 |
| | claudin-c | −0.963 | < .01 | −0.969 | < .01 | −0.976 | < .01 |
| | claudin-f | −0.992 | < .01 | −0.976 | < .01 | −0.986 | < .01 |
| | claudin-3c | −0.976 | < .01 | −0.908 | < .05 | −0.998 | < .01 |
| | claudin-7a | −0.940 | < .01 | −0.794 | = .059 | – | – |
| | claudin-7b | −0.932 | < .05 | −0.943 | < .01 | −0.925 | < .01 |
| | claudin-11 | −0.897 | < .05 | −0.928 | < .01 | −0.952 | < .01 |
| | claudin-12 | +0.964 | < .01 | +0.933 | < .01 | +0.986 | < .01 |
| caspase-8 | FasL | – | – | – | – | +0.986 | < .01 |
| | TNF-α | +0.983 | < .01 | +0.943 | < .01 | +0.801 | = .056 |
| caspase-9 | Apaf-1 | +0.938 | < .01 | +0.969 | < .01 | +0.974 | < .01 |
| | Bax | +0.979 | < .01 | +0.986 | < .01 | +0.973 | < .01 |
| | Bcl-2 | −0.949 | < .01 | −0.928 | < .01 | −0.989 | < .01 |
| | Mcl-1 | −0.881 | < .05 | −0.947 | < .01 | −0.987 | < .01 |
| | IAP | −0.979 | < .01 | −0.967 | < .01 | −0.992 | < .01 |
| caspase-3 | caspase-8 | +0.943 | < .01 | +0.991 | < .01 | +0.896 | < .05 |
| | caspase-9 | +0.927 | < .01 | +0.983 | < .01 | +0.987 | < .01 |
| caspase-7 | caspase-8 | +0.998 | < .01 | +0.952 | < .01 | +0.955 | < .01 |
| | caspase-9 | +0.990 | < .01 | +0.989 | < .01 | +0.963 | < .01 |
| JNK | FasL | – | – | – | – | +0.871 | < .05 |
| | TNF-α | +0.904 | < .01 | +0.983 | < .01 | +0.978 | < .01 |
| | Apaf-1 | +0.899 | < .05 | +0.968 | < .01 | +0.950 | < .01 |
| | Bax | +0.949 | < .01 | +0.985 | < .01 | +0.935 | < .01 |
| | Bcl-2 | −0.976 | < .01 | −0.916 | < .01 | −0.974 | < .01 |
| | Mcl-1 | −0.882 | < .05 | −0.916 | < .01 | −0.974 | < .01 |
| | IAP | −0.881 | < .05 | −0.962 | < .01 | −0.995 | < .01 |

(*continued on next page*)

**Table 3** (*continued*)

| Independent parameters | Dependent parameters | PI | | MI | | DI | |
|---|---|---|---|---|---|---|---|
| | | Correlation coefficients | *P* | Correlation coefficients | *P* | Correlation coefficients | *P* |
| Nrf2 | CuZnSOD | +0.987 | < .01 | +0.923 | < .01 | +0.959 | < .01 |
| | MnSOD | +0.961 | < .01 | – | – | +0.976 | < .01 |
| | CAT | +0.995 | < .01 | +0.979 | < .01 | +0.987 | < .01 |
| | GPx1a | +0.993 | < .01 | +0.922 | < .01 | +0.929 | < .01 |
| | GPx1b | +0.992 | < .01 | +0.991 | < .01 | +0.978 | < .01 |
| | GSTR | +0.986 | < .01 | +0.913 | < .05 | +0.955 | < .01 |
| | GSTO1 | +0.989 | < .01 | +0.900 | < .05 | +0.962 | < .01 |
| | GSTP1 | +0.982 | < .05 | +0.986 | < .01 | +0.972 | < .01 |
| | GR | +0.972 | < .01 | +0.931 | < .01 | +0.944 | < .01 |
| | Keap1a | −0.973 | < .01 | −0.952 | < .01 | – | – |
| | Keap1b | −0.814 | < .05 | −0.810 | = .051 | – | – |

**Table 4**
The available phosphorus (AP) requirements based on enteritis morbidity, LZ and ACP activities and ROS and MDA contents in the proximal intestine (PI) of grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of available phosphorus.

| Indices | Regressive equation | | $R^2$ | *P* | AP requirement |
|---|---|---|---|---|---|
| Enteritis morbidity | $Y = -5.4644x + 39.7946$ | $Y_{min} = 14.22$ | 0.6568 | < .01 | 4.68 g/kg |
| LZ | $Y = 28.8475x + 51.1817$ | $Y_{max} = 201.71$ | 0.8765 | < .01 | 5.22 g/kg |
| ACP | $Y = -9.4851x^2 + 105.7323x + 143.9312$ | | 0.7458 | < .01 | 5.57 g/kg |
| ROS | $Y = 2.1886x^2 - 25.2889x + 123.2191$ | | 0.9008 | < .01 | 5.78 g/kg |
| MDA | $Y = -2.9097x + 35.8687$ | $Y_{min} = 19.31$ | 0.8238 | < .01 | 5.69 g/kg |

(1) phosphorus deficiency decreased LZ and ACP activities, C3 and C4 contents, as well as down-regulated antimicrobial peptides (except hepcidin) LEAP-2A, LEAP-2B, β-defensin-1 and Mucin2 mRNA levels; (2) phosphorus deficiency down-regulated the anti-inflammatory cytokines IL-4/13A, IL-4/13B, IL-10, IL-11, TGF-β1 and TGF-β2 and up-regulated the pro-inflammatory cytokines IL-1β (not in DI), IL-6, IL-8, IL-12p35 (not in DI), IL-15, IL-17D, TNF-α and IFN-γ2 mRNA levels, which may be involved in TOR/(S6K1, 4E-BP) and IKKβ, IKKγ (rather than IKKα)/IκBα/NF-κB signalling pathways, respectively. In addition, fish intestinal physical barrier, composed of intercellular TJs and epithelial cells, is of vital importance for fish intestinal health [2,82]. Thus, we further elucidated the effects of phosphorus deficiency on the intercellular TJs and epithelial cells in the intestine of grass carp.

### *4.3. Phosphorus deficiency impaired intestinal physical barrier function partly relating to MLCK, JNK and Nrf2 signalling in fish*

#### *4.3.1. Phosphorus deficiency disturbed the TJs partly by MLCK signalling in the intestine of fish*

In fish, the intestinal physical barrier tightly linked to tight junction complexes which is primarily composed of ZO, occludin, barrier-forming TJs claudins and pore-forming TJs like claudin-12 [83]. A study demonstrated that MLCK was a centre to regulate TJs expression in human Caco-2 cells [84]. In the study, compared with optimal phosphorus level, phosphorus deficiency down-regulated ZO-1, ZO-2, occludin (only in DI), claudin-b, -c, -f, -3c, -7a, -7b and −11 mRNA levels and up-regulated pore-forming TJ claudin-12 and MLCK mRNA levels in the three intestinal segments of grass carp. Correlation analysis showed that these TJs mRNA levels were negatively related to the MLCK mRNA levels in the three intestinal segments of grass carp (Table 3), suggesting that the decreased of TJs expression by phosphorus deficiency might be in part owed to the increased of MLCK expression to disturbed intercellular TJs in the three intestinal segments of fish.

Interestingly, dietary phosphorus up-regulated occludin mRNA levels only in the DI, which might be partially explained two ways. First, it might be related to intestinal flora. Corridoni et al. reported that probiotic bacteria containing *Lactobacillus* increased occludin mRNA level in mice ilea [85]. The previous study showed that dietary phosphorus increased the *Lactobacillus* count in the intestine of Jian carp [35]. Second, in fish, the contents of harmful bacteria and toxins in the DI higher than that in the PI and MI [2]. Thus, dietary phosphorus might increase occludin mRNA level in the DI (rather than PI and MI) to enhance barrier function and defend harmful bacteria and toxins in fish. However, this presumption warrants investigation. In addition, we surprisingly found that phosphorus deficiency both had no effects on claudin-15a and −15b mRNA levels in the three intestinal segments of grass carp. In our previous study, it was reported that phosphorus deficiency only had no effects on claudin-15b in the head kidney and spleen [20]. This interesting phenomena might be partially related to the transcript abundance of NaPi-IIb in different tissue. In fish, the transcript abundance of NaPi-IIb in intestine higher than that in other organs (such as kidney and spleen) [86]. NaPi-IIb and claudin-15 both could take part in transporting $Na^+$ in fish [87,88]. Thus, because of higher transcript abundance of NaPi-IIb in intestine than that in head kidney and spleen, we hypothesize that NaPi-IIb might completely replace the function of claudin-15 leading to unaffected mRNA levels of claudin-15a and −15b in the three intestinal segments, whereas NaPi-IIb might be partly replace that function in the head kidney and spleen of fish and showed us the interesting results. Certainly, the hypothesis awaits further characterization. Besides intercellular TJs, the integrity of epithelial cells is also critical for intestinal physical barrier, which could be disturbed by apoptosis and oxidative damage in fish [43]. Accordingly, we firstly examined the effects of phosphorus deficiency on apoptosis in the intestine of grass carp.

#### *4.3.2. Phosphorus deficiency induced apoptosis partly referring to JNK signalling in the intestine of fish*

It has been reported that apoptotic pathways are mainly classified into the mitochondrial pathway and the death ligand pathway, and caspases are central effectors of apoptosis in mammals [89]. The death receptor pathway is primarily regulated by FasL/TNF-α and caspase-8 in mammals [90]. JNK could active TNF-α and FasL to induce apoptosis in mice [91]. In our study, compared with optimal phosphorus level, phosphorus deficiency up-regulated the mRNA levels of TNF-α, caspase-8 and JNK in the three intestinal segments and pro-apoptotic factor FasL only in the DI. Correlation analysis displayed that TNF-α mRNA levels were positively related to caspase-8 and JNK in the three

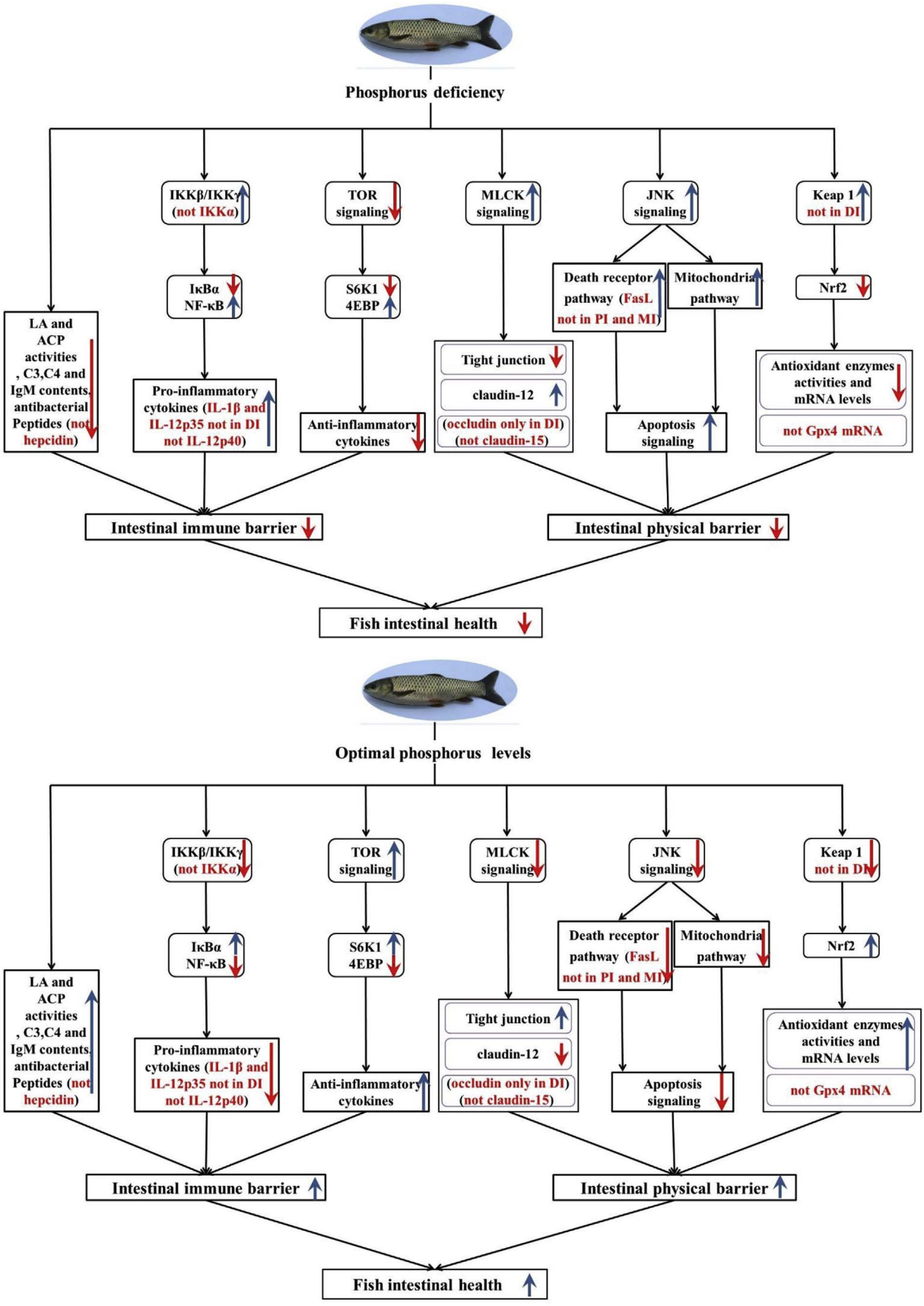


**Fig. 5.** The potential action pathways of phosphorus deficiency and optimal phosphorus level in the intestinal immune barrier and physical barrier function of fish.

intestinal segments of grass carp, and FasL mRNA level was positively related to caspase-8 and JNK in the DI of grass carp (Table 3). These results indicated that phosphorus deficiency could induce apoptosis partly referring to JNK/TNF-α/caspase-8 signalling in the three intestinal segments and JNK/FasL/caspase-8 signalling in the DI of fish.

Interestingly, phosphorus deficiency up-regulated FasL gene expression in the DI (rather than PI and MI), which might be correlated with glucocorticoid receptor. It has been demonstrated that chronic phosphorus deficiency decreased ATP concentration in mammals [6], and a low concentration of ATP inhibited the activation of glucocorticoid receptor in mouse Sf9 cell [92]. Studies reported that inhibited glucocorticoid receptor up-regulated FasL mRNA level in Jurkat T cells [93], and its mRNA abundance was predominant in the DI of Mozambique tilapia (*Oreochromis mossambicus*) [94]. According to above data, we suppose that phosphorus deficiency might decrease ATP concentration leading to the decline of glucocorticoid receptor activation and then up-regulated FasL mRNA level in the DI (rather than PI and MI) of fish. However, the supposition warrants further investigation.

The mitochondrial apoptotic pathway [(Bcl-2, Mcl-1 and Bax)/Apaf-1/caspase-9] could affect cell apoptosis in mammals [89]. JNK activated mitochondrial apoptotic pathway through the phosphorylation of Bcl-2 and Bax in murine embryonic fibroblasts [95]. IAP was an anti-apoptotic regulator that block cell death in response to diverse stimuli in human [96]. In the present study, compared with optimal phosphorus level, phosphorus deficiency up-regulated the mRNA levels of caspase-3, -7, -9, pro-apoptotic factors Bax and Apaf-1 as well as down-regulated the mRNA levels of anti-apoptotic factors Bcl-2, Mcl-1 and IAP in the three intestinal segments of grass carp. A further correlation analysis found that caspase-3 and -7 mRNA levels were positively related to caspase-9 mRNA levels, and caspase-9 as well as JNK mRNA levels were positively correlated with pro-apoptotic factors Bax and Apaf-1 mRNA levels and negatively related to anti-apoptotic factors Bcl-2, Mcl-1 and IAP mRNA levels in the three intestinal segments of grass carp (Table 3). According to the above results, phosphorus deficiency could induce apoptosis partly relating to mitochondrial apoptotic pathway [JNK/(Bcl-2, Mcl-1 and Bax)/Apaf-1/caspase-9/caspase-3, -7] in the intestine of fish.

#### *4.3.3. Phosphorus deficiency induced oxidative damage and decreased antioxidant capacity partly relating to Nrf2 signalling in the intestine of fish*

Under the condition of stress, organisms would produce free radical leading to oxidative damage, which could be relieved by enzymatic and non-enzymatic antioxidants in fish [97]. In the study, compared with the optimal phosphorus level, phosphorus deficiency increased ROS, MDA and PC contents and decreased the CuZnSOD, MnSOD, CAT, GPx, GST and GR activities and GSH contents in the three intestinal segments of grass carp, suggesting that phosphorus deficiency decreased the intestinal antioxidant capacity and induced intestinal oxidative damage of fish. To our knowledge, antioxidant enzymes activities are partly relies on their mRNA levels in fish [98]. Therefore, we next investigated the relationship between phosphorus and antioxidant enzymes gene transcriptions in the intestine of grass carp. Our data showed that compared with optimal phosphorus level, phosphorus deficiency down-regulated CuZnSOD, MnSOD, CAT, GPx (1a, 1b), GST (GSTR, P, O) and GR mRNA levels in the three intestinal segments, which showed similar patterns compared with the activities of those antioxidant activities, implying that phosphorus deficiency decreased the intestinal antioxidant activities partially referring to the down-regulated of their mRNA levels in the intestine of fish.

Interestingly, phosphorus deficiency down-regulated GPx1 mRNA levels, but had no impacts on GPx4 in the three intestinal segments of grass carp, which might be partly related to dopamine regulated by TOR. In male C57/BL mice, the inhibition of mTOR decreased striatum dopamine level [99]. Ma et al. reported that inhibited dopamine decreased GPx1 (rather than GPx4) level in human SH-SY5Y cell [100]. In our study, phosphorus deficiency down-regulated TOR mRNA levels in the three intestinal segments of grass carp. Hence, we hypothesize that phosphorus deficiency down-regulated TOR mRNA levels leading to the decline of dopamine levels, which might down-regulate GPx1 (rather than GPx4) mRNA levels in the three intestinal segments of fish. However, this hypothesis requires deeper investigation. Moreover, of note in present study, phosphorus deficiency decreased CAT activities in the intestine of grass carp, while in our previous study, phosphorus deficiency couldn't change CAT activity in the spleen [20]. The interesting results might be an adaptive mechanism. To our knowledge, CAT and GPx have the same function of the elimination of $H_2O_2$ in fish [101]. Compared with spleen, the activities of GPx in the intestine (96.84–162.90 U/mg protein) lower than that in spleen (213.54–289.47 U/mg protein). Therefore, we suppose that lower GPx activities in the intestine might need CAT to take part in the elimination of $H_2O_2$, while higher GPx activity in spleen might completely replace the function of CAT resulting in this interesting phenomenon of fish. However, a further exploration should be conducted to verify our supposition.

Nrf2 could be activated through Keap1 releasing in response to a range of oxidative and electrophilic stimuli by ROS in fish [102]. In our study, compared with the optimal phosphorus level, phosphorus deficiency down-regulated Nrf2 mRNA levels in the three intestinal segments and up-regulated Keap1a and Keap1b mRNA levels in the PI and MI of grass carp. Correlation analysis showed that CuZnSOD, MnSOD, CAT, GPx (1a, 1b), GST (GSTR, P, O) and GR mRNA levels were positively related to Nrf2 mRNA levels in the three intestinal segments which were negatively related to Keap1a and Keap1b mRNA levels in the PI and MI of grass carp (Table 3). These data indicated that phosphorus deficiency down-regulated antioxidant enzymes mRNA levels partially relating to Keap1/Nrf2 signalling in the PI and MI of fish. Interestingly, dietary phosphorus down-regulated Keap1 mRNA levels in the PI and MI, but had no influences on Keap1 mRNA levels in the DI of grass carp. To date, no reports have shown the relationship between dietary phosphorus and Keap1 mRNA levels in fish intestine. Above results might be related to folic acid in fish. It was reported that phosphorus metabolite GTP-Cyclohydrolase I could increase folic acid synthesis [103]. A previous study from our lab found that folic acid down-regulated Keap1 mRNA levels in the intestine of grass carp [83], and a report revealed that the folic acid transporter is expressed mainly in the proximal part of the human small intestine, with low expression in the distal small intestine [104]. Thus, we suppose that phosphorus might indirectly increase folic acid contents leading to down-regulated of Keap1 mRNA level in the PI and MI (rather than DI) in fish. However, our supposition should further investigate.

#### *4.3.4. Brief summary: phosphorus deficiency impaired intestinal physical barrier function in different intestinal segments of grass carp*

In summary, the data above indicated that phosphorus deficiency impaired intestinal physical barrier function in the three intestinal segments of grass carp, which is associated with intercellular TJs, apoptosis and oxidative damage and could be displayed as follow: (1) phosphorus deficiency down-regulated the TJs (except claudin-15) ZO-1, ZO-2, occludin (not in DI), claudin-b, -c, -f, -3c, -7a, -7b and −11 mRNA levels partially correlating with MLCK to disturbed intercellular TJs; (2) phosphorus deficiency aggravated death receptor pathway TNF-α/FasL (FasL in the DI)/caspase-8 and mitochondria pathway [(Bcl-2, Mcl-1 and Bax)/Apaf-1/caspase-9] partly relating to JNK to induce apoptosis; (3) phosphorus deficiency increased ROS, PC and MDA contents, and decreased antioxidant enzymes activities and mRNA levels (expect GPx4) partially associating with Keap1 (not in DI)/Nrf2 signalling to induce oxidative damage.

### *4.4. Dietary available phosphorus requirements for grass carp*

It is necessary to estimate the nutritional requirements by sensitive

biomarkers in animals [105]. Studies showed that LZ and ACP are the sensitive indicators of immune [43], and ROS and MDA are the sensitive indicators of oxidative damage in fish [20,83]. The available phosphorus requirement for the ability to combat enteritis was estimated to be 4.68 g/kg diet (Table 4). Additionally, according to immune indices (LZ and ACP activities in the PI) and antioxidant indices (ROS and MDA contents in the PI), the available phosphorus requirements of grass carp were estimated to be 5.22, 5.57, 5.78 and 5.69 g/kg diet, respectively. The available phosphorus requirements for the ability to combat enteritis and intestinal immune and antioxidant indices of fish are higher than that for growth performance (4.10 g/kg diet) proposed by our previous study [20]. This phenomenon might be explained by the extensive metabolite of phosphorus (such as ATP, DNA, RNA, phospholipids and so on), which could take part in a series of metabolisms and physiological and biochemical reactions in fish [7].

## 5. Conclusions

Summarily (Fig. 5), the current study provided evidences that dietary phosphorus deficiency impaired intestinal immune barrier and physical barrier function of fish, and provided partial theoretical evidence for the underlying molecular mechanisms. The three primary results of this study were showed for the first time. (1) Phosphorus deficiency impaired the intestinal immune barrier function by causing the decline of antimicrobial function and aggravated of inflammatory responses which might be related to the TOR and NF-κB signalling pathways. (2) Phosphorus deficiency disturbed intercellular TJs, aggravated cell apoptosis and induced oxidative damage leading to the impaired of intestinal physical barrier function, which might be partially associated with the MLCK, JNK and Nrf2 signalling pathways, respectively. (3) Based on the ability to combat enteritis, dietary available phosphorus requirement for grass carp (254.56–898.23 g) was estimated to be 4.68 g/kg diet.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.12.060.

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