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Vitamin A deficiency impairs intestinal physical barrier function of fish

Wei-Dan Jiang[a,b,c,1], Xiao-Qiu Zhou[a,b,c,1], Li Zhang[a,1], Yang Liu[a,b,c], Pei Wu[a,b,c], Jun Jiang[a], Sheng-Yao Kuang[d], Ling Tang[d], Wu-Neng Tang[d], Yong-An Zhang[e], He-Qun Shi[f], Lin Feng[a,b,c,*]

[a] Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, 611130, China
[b] Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu, 611130, China
[c] Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Sichuan Agricultural University, Chengdu, 611130, China
[d] Animal Nutrition Institute, Sichuan Academy of Animal Science, Chengdu, 610066, China
[e] Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, China
[f] Guangzhou Cohoo Bio-tech Research & Development Centre, Guangzhou, 510663, Guangdong, China

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ABSTRACT

The present study was the first to investigate the effects of dietary vitamin A (VA) on the intestinal physical barrier function associated with oxidation, antioxidant system, apoptosis and cell-cellular tight junction (TJ) in the proximal (PI), mid (MI) and distal (DI) intestines of young grass carp (*Ctenopharyngodon idella*). Fish were fed graded levels of dietary VA for 10 weeks, and then a challenge test using an injection of *Aeromonas hydrophila* was conducted for 14 days. Results indicated that dietary VA deficiency caused oxidative damage to fish intestine partly by the reduced non-enzymatic antioxidant components glutathione (GSH) and VA contents as well as reduced antioxidant enzyme activities [not including manganese superoxide dismutase (MnSOD)]. Further results observed that the decreased antioxidant enzyme activities by VA deficiency were partly related to the down-regulation of their corresponding mRNA levels which were regulated by the down-regulation of *NF-E2-related factor 2 (Nrf2)* mRNA levels and up-regulation of *kelch-like-ECH-associated protein (Keap1a)* (rather than *Keap1b*) mRNA levels in three intestinal segments of fish. Meanwhile, VA deficiency up-regulated the mRNA levels of the apoptosis signalling [*caspase-3*, *caspase-8*, *caspase-9* (rather than *caspase-7*)] associated with the inhibition of the target of rapamycin (TOR) signalling pathway in three intestinal segments of fish. Additionally, VA deficiency decreased the mRNA levels of TJ complexes [*claudin-b, claudin-c, claudin-3, claudin-12, claudin-15a, occludin* and *zonula occludens-1 (ZO-1)* in the PI, MI and DI, as well as claudin-7 and claudin-11a in the MI and DI] linked to the up-regulation of *myosin light chain kinase (MLCK)* signalling. These results suggested that VA deficiency impaired structural integrity in three intestinal segments of fish. Meanwhile, excessive VA also showed similar negative effects on these indexes. Taken together, the current study firstly demonstrated that VA deficiency impaired physical barrier functions associated with impaired antioxidant capacity, aggravated cell apoptosis and disrupted TJ complexes in the PI, MI and DI, but different segments performed different actions in fish. Based on protecting fish against protein oxidation, the optimal VA levels for grass carp were estimated to be 2622 IU/kg diet.

1. Introduction

The intestine of animals is an important physical barrier against pathogen entry [1]. However, it is well known that animal intestines with high levels of polyunsaturated fatty acids (PUFAs) are sensitive to oxidative damage [2,3]. Meanwhile, the intestine is frequently exposed to the reactive oxygen species (ROS) produced from ingested potential pathogens in animals, which increases the chance of oxidative damage [4]. Studies observed that oxidative damage could result in cell apoptosis of C2C12 cells [5] and disruption of cell-cellular tight junctions (TJs) in Sertoli cells [6]. Hence, enhancement of the antioxidant ability and maintaining the structural integrity of the intestine is of utmost importance to the health of animals. Our previous study observed that vitamins, such as thiamin, could enhance the antioxidant ability and inhibit oxidative damage in the intestine of young grass carp (*Ctenopharyngodon idella*) [7]. Vitamin A (VA) is an essential vitamin for the normal growth of animals [8]. Our previous study observed that VA deficiency impaired the intestinal immunity and depressed the growth of

* Corresponding author. Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, 611130, Sichuan, China.
E-mail address: fenglin@sicau.edu.cn (L. Feng).
[1] These three authors contributed to this work equally.

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young grass carp [9]. Intestinal immunity largely relies on the structural integrity of intestine in animals [10]. However, no reports at present have addressed the effects of VA on the structural integrity of the intestine in animals. Nikawa et al. (1999) reported that dietary VA supplementation could increase VA content in the small intestine of mice [11]. Meanwhile, it was proven that VA could improve the balance of intestinal bacteria in rats [12]. In general, intestinal bacteria were closely associated with intestinal structural integrity in fish [13]. Those observations implied a potential relationship between VA and intestinal structural integrity in animals, which warrants further investigation.

The structural integrity of animal intestine largely relies on the cellular structural integrity [14]. To our knowledge, cellular antioxidant ability and apoptosis play important roles in the cellular structural integrity in animals [15]. Studies have shown that the antioxidant system and apoptosis could be regulated by signalling molecules NF-E2-related factor 2 (Nrf2) [16] and target of rapamycin (TOR) [17] in zebrafish, respectively. However, the relationship between VA and the intestinal cellular structural integrity as well as its mechanisms in animals are unclear. VA deficiency has been reported to lead to oxidative damage in the livers of rats [18]. Rojo et al. (2007) found that oxidative damage could decrease Nrf2 transcriptional activity in rats [19]. Meanwhile, VA deficiency caused up-regulation of the expression of tumour-suppressor protein 53 (p53) in the livers of rats [20]. To our knowledge, the activation of p53 could result in cell apoptosis in humans [21] and down-regulate TOR signalling in mouse embryo fibroblast (MEF) cells [22]. Those data indicate that VA deficiency may influence the cellular structural integrity associated with oxidative damage and cell apoptosis as well as the related signalling pathways in the intestines of animals, which requires investigation.

In addition to cellular structural integrity, cell-to-cell TJs also play important roles in the structural integrity of fish intestines. It is well known that TJs are comprised of transmembrane (such as occludin and claudins) and cytoplasmic [such as zonula occludens-1 (ZO-1)] proteins, which could be regulated by signalling molecule myosin light chain kinase (MLCK) in fish [23]. However, to date, no study has addressed the effect of VA on TJ proteins and the related signalling pathways in animal intestines. Varani et al. (2000) found that VA could reduce the levels of matrix metalloproteinases (MMP) in human skin [24]. It was reported that inhibition of MMP activity could promote the expression of the TJ protein ZO-1 and occludin in rat brain [25]. Besides, it was reported that VA could increase androgen production in rats [26]. Study found that androgen was able to down-regulate MLCK mRNA levels in human prostate cancer cells [27]. These data reveal a potential relationship between VA and fish intestinal physical barrier associated with TJs as well as related signalling pathways, which is worth of investigations.

This study used the same growth trial as our previous study, which determined that 0.664 mg/kg of VA could improve growth of young grass carp [9]. In the present study, we propose a hypothesis that dietary VA deficiency might impair fish intestinal physical barrier functions. To test this hypothesis, we are for the first time to investigate effects of VA on apoptosis signalling, antioxidant defence and TJ complexes in three intestinal segments of fish challenged with *Aeromonas hydrophila*. Furthermore, through investigating effects of VA on the related signalling molecules such as TOR, Nrf2 and MLCK, we may provide partial theoretical evidences for the mechanisms about the effects of VA on physical barrier functions in the different intestinal segments of animals.

2. Materials and methods

2.1. Experimental diet preparation and experiments

The formulation of the basal diet was the same as our previous study [9] and is presented in Table S1. Different concentrations of retinyl acetate (500,000 IU/g) were added to a basal diet to constitute the six levels of 0 (un-supplemented control), 600, 1200, 1800, 2800 and 3800 IU/kg diet, and the amount of corn starch was reduced to compensate. The final VA concentrations in each experimental diet were analyzed according to Moren et al. [28] to be 18.69 (un-supplemented control), 606.8, 1209, 1798, 2805 and 3796 IU/kg diet. All ingredients were mixed, pelleted and stored at −20 °C until to be used, which was similar to the method of Guimarães et al. [29].

After an acclimatization period of 4 weeks as described by Shaik Mohamed et al. [30], the fish were fed with the VA-deficient diet for 2 weeks to diminish the body storage of VA according to Campeche et al. [31]. Thereafter, 540 fish (mean weight 262.02 ± 0.45 g) were randomly distributed into 18 experimental cages (1.4 L × 1.4 W × 1.4H m), namely 30 fish per cage. Furthermore, each cage was equipped with a disc (100 cm diameter) in the bottom to collect the uneaten feed as described by our previous study [32]. The experiment design included 6 treatments with 3 replications, feeding with the respective diet four times daily for 10 weeks. Fish were fed with the respective diet to apparent satiation, and after feeding 30 min, uneaten feed was collected according to the method of Mundheim et al. [33]. Water temperature was determined to be 28 ± 2 °C throughout the experiment. The pH and dissolved oxygen were maintained at 7.0 ± 0.2 and not less than 6.0 mg/L, respectively, and the experimental units were under natural light and dark cycle, similar to Luo et al. [32].

At the end of the growth trial, a challenge trial was conducted according to the same method as our previous study [9]. Briefly, 15 fish from each treatment were randomly collected with similar body weights and moved into labelled cages. After 5-day acclimation, fish were intraperitoneally injected with *Aeromonas hydrophila* (friendly provided by College of Veterinary Medicine, Sichuan Agricultural University) at a dose of 2.5×10^8 colony-forming units (cfus)/mL for each individual. The challenge test was conducted for 14 d. Experiment conditions were the same as the growth trial. After completion of the challenge experiment, all fish from each treatment were anaesthetized in a benzocaine bath according to Chen et al. [34]. After sacrificing, fish intestines were quickly removed, stored on ice and divided into proximal (PI), mid (MI) and distal (DI) intestines according to the turning point described by Askarian et al. [35]. Parts of the intestine were preserved in 10% neutral buffered formalin for histological examination as described by our laboratory previous study [36]. The morphological changes were scored as follows: 0 = not observed, 1 = low (1–3 out of 10 images), 2 = moderate (4–6 out of 10 images), and 3 = high (7 or more out of 10 images) according the method described by Liu et al. (2016) [37]. The rest of the intestines were frozen in liquid nitrogen, and stored at −80 °C until the subsequent analysis, according to descriptions by Martins et al. [38].

2.2. Biochemistry assay

The intestinal sample was homogenized on ice in 10 vol (w/v) of sterile ice-cold physiological saline (0.9% NaCl) and centrifuged at 6000 g at 4 °C for 20 min. Then, the collected supernatant was stored at −20 °C as described by Kuang et al. [39] for the subsequent related parameters analysis. The reactive oxygen species (ROS) and protein carbonyl (PC) contents using the 2,4-dinitrophenylhydrazine reagent were determined by the method of Chen et al. [34]. Anti-superoxide anion (ASA) and anti-hydroxyl radical (AHR) capacities were measured on the basis of Hong et al. [40]. The activities of catalase (CAT), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST) and glutathione reductase (GR) were determined as described by Peixoto et al. [41]. Analyses of superoxide dismutase (SOD) activity, malondialdehyde (MDA) and reduced glutathione (GSH) contents were performed following the method of Zhang et al. [42]. Briefly, malondialdehyde (MDA) equivalents were measured using the thiobarbituric acid (TBA) reaction. For CuZnSOD, the reaction mixture contained 50 mM of phosphate buffer (pH 7.8), 1.08 mM of

Table 1
Effects of vitamin A (VA) on the condition factor, oxidative status, antioxidant abilities and VA contents in the proximal (PI), mid (MI) and distal (DI) intestines of young grass carp (*Ctenopharyngodon idella*).

	Dietary VA levels (IU/kg diet)					
	18.69	606.8	1209	1798	2805	3796
CF	1.79 ± 0.07^{a}	1.82 ± 0.08^{ab}	1.85 ± 0.04^{b}	1.86 ± 0.05^{b}	1.81 ± 0.11^{ab}	1.77 ± 0.06^{a}
PI						
MDA	6.26 ± 0.39^{d}	5.79 ± 0.24^{c}	4.93 ± 0.26^{a}	4.63 ± 0.30^{a}	5.38 ± 0.33^{b}	5.35 ± 0.26^{b}
PC	11.21 ± 0.23^{e}	8.08 ± 0.39^{d}	7.33 ± 0.56^{c}	4.56 ± 0.32^{a}	5.79 ± 0.45^{b}	5.85 ± 0.37^{b}
AHR	51.53 ± 1.97^{a}	51.16 ± 3.44^{a}	52.96 ± 2.01^{a}	52.90 ± 3.92^{a}	51.52 ± 4.33^{a}	51.10 ± 3.02^{a}
ASA	57.49 ± 4.91^{a}	74.43 ± 6.75^{b}	86.30 ± 4.53^{c}	85.15 ± 5.00^{c}	85.02 ± 3.29^{c}	83.84 ± 4.0^{c}
T-SOD	7.75 ± 0.16^{a}	8.04 ± 0.27^{a}	9.36 ± 0.57^{c}	9.92 ± 0.66^{c}	9.54 ± 0.55^{c}	8.69 ± 0.53^{b}
CuZnSOD	5.41 ± 0.20^{a}	5.70 ± 0.17^{a}	6.97 ± 0.46^{c}	7.40 ± 0.50^{c}	7.13 ± 0.66^{c}	6.34 ± 0.54^{b}
MnSOD	2.34 ± 0.18^{a}	2.34 ± 0.17^{a}	2.39 ± 0.21^{a}	2.52 ± 0.21^{a}	2.41 ± 0.13^{a}	2.35 ± 0.13^{a}
CAT	1.58 ± 0.12^{a}	1.98 ± 0.09^{bc}	2.09 ± 0.09^{c}	2.02 ± 0.09^{bc}	1.98 ± 0.12^{bc}	1.95 ± 0.12^{b}
GPx	43.38 ± 1.95^{a}	51.66 ± 3.13^{b}	56.61 ± 4.30^{b}	72.38 ± 6.09^{c}	73.24 ± 5.11^{c}	75.33 ± 3.76^{c}
GST	42.91 ± 3.77^{a}	46.42 ± 4.31^{ab}	49.08 ± 3.39^{bc}	52.73 ± 4.49^{c}	48.49 ± 2.97^{bc}	47.15 ± 4.28^{ab}
GR	21.40 ± 1.07^{a}	29.60 ± 3.22^{b}	36.40 ± 1.76^{c}	43.81 ± 3.43^{d}	40.63 ± 3.50^{d}	31.94 ± 2.59^{b}
GSH	4.07 ± 0.07^{a}	4.93 ± 0.33^{b}	5.65 ± 0.42^{c}	5.53 ± 0.24^{c}	4.42 ± 0.36^{a}	4.39 ± 0.24^{a}
VA contents	4.18 ± 0.36^{a}	4.32 ± 0.33^{a}	4.90 ± 0.34^{b}	5.50 ± 0.25^{c}	5.57 ± 0.25^{c}	5.64 ± 0.27^{c}
MI						
MDA	4.36 ± 0.34^{e}	3.49 ± 0.20^{d}	2.07 ± 0.10^{a}	2.55 ± 0.21^{b}	2.76 ± 0.15^{b}	3.05 ± 0.20^{c}
PC	7.74 ± 0.45^{c}	6.10 ± 0.45^{a}	6.30 ± 0.33^{a}	6.21 ± 0.21^{a}	6.57 ± 0.38^{ab}	7.01 ± 0.43^{b}
AHR	55.81 ± 2.47^{a}	55.77 ± 3.73^{a}	55.29 ± 3.40^{a}	55.13 ± 3.31^{a}	55.70 ± 3.17^{a}	55.60 ± 4.16^{a}
ASA	73.45 ± 4.98^{a}	74.97 ± 4.26^{a}	96.16 ± 6.55^{b}	105.94 ± 5.48^{c}	98.26 ± 7.73^{b}	93.47 ± 2.58^{b}
T-SOD	8.70 ± 0.40^{a}	9.70 ± 0.32^{b}	12.22 ± 0.31^{e}	11.30 ± 0.48^{d}	11.11 ± 0.85^{d}	10.49 ± 0.34^{c}
CuZnSOD	5.49 ± 0.30^{a}	6.16 ± 0.38^{b}	8.71 ± 0.30^{e}	7.99 ± 0.25^{d}	7.90 ± 0.66^{d}	7.21 ± 0.38^{c}
MnSOD	3.21 ± 0.16^{a}	3.54 ± 0.26^{a}	3.51 ± 0.29^{a}	3.31 ± 0.33^{a}	3.21 ± 0.25^{a}	3.27 ± 0.25^{a}
CAT	1.23 ± 0.12^{a}	1.31 ± 0.05^{a}	1.59 ± 0.11^{b}	1.90 ± 0.18^{c}	1.87 ± 0.10^{c}	1.56 ± 0.14^{b}
GPx	42.33 ± 3.44^{a}	49.89 ± 2.26^{b}	89.55 ± 6.75^{e}	85.54 ± 5.66^{e}	74.45 ± 5.16^{d}	60.72 ± 3.63^{c}
GST	44.17 ± 2.71^{a}	49.98 ± 2.48^{b}	54.64 ± 3.68^{c}	56.83 ± 4.33^{c}	49.33 ± 2.56^{b}	50.02 ± 3.35^{b}
GR	25.16 ± 2.50^{a}	28.93 ± 2.51^{b}	38.88 ± 1.71^{d}	39.05 ± 2.16^{d}	44.61 ± 1.89^{e}	35.52 ± 1.42^{c}
GSH	5.02 ± 0.27^{a}	5.26 ± 0.37^{ab}	5.52 ± 0.30^{bc}	8.73 ± 0.33^{e}	6.87 ± 0.34^{d}	5.82 ± 0.29^{c}
VA contents	2.44 ± 0.21^{a}	2.62 ± 0.12^{b}	2.81 ± 0.06^{c}	3.05 ± 0.11^{d}	3.17 ± 0.14^{d}	3.19 ± 0.14^{d}
DI						
MDA	3.67 ± 0.14^{c}	3.53 ± 0.19^{c}	2.16 ± 0.16^{a}	2.26 ± 0.19^{a}	2.97 ± 0.16^{b}	3.15 ± 0.20^{b}
PC	7.76 ± 0.24^{c}	5.65 ± 0.43^{a}	5.76 ± 0.49^{a}	5.58 ± 0.27^{a}	7.02 ± 0.33^{b}	7.09 ± 0.45^{b}
AHR	55.12 ± 3.13^{a}	55.42 ± 1.43^{a}	55.35 ± 3.13^{a}	56.06 ± 1.77^{a}	55.85 ± 3.99^{a}	55.67 ± 3.63^{a}
ASA	78.08 ± 5.47^{a}	94.36 ± 7.89^{b}	116.86 ± 7.67^{c}	128.18 ± 8.08^{d}	118.06 ± 9.33^{c}	99.64 ± 2.72^{b}
T-SOD	11.64 ± 0.35^{a}	12.92 ± 0.57^{b}	15.43 ± 0.68^{d}	14.03 ± 0.62^{c}	13.34 ± 0.67^{b}	12.98 ± 0.50^{b}
CuZnSOD	8.42 ± 0.42^{a}	9.67 ± 0.54^{b}	12.13 ± 0.72^{d}	10.78 ± 0.54^{c}	10.07 ± 0.69^{b}	9.73 ± 0.53^{b}
MnSOD	3.22 ± 0.27^{a}	3.26 ± 0.23^{a}	3.29 ± 0.16^{a}	3.25 ± 0.11^{a}	3.28 ± 0.18^{a}	3.25 ± 0.22^{a}
CAT	1.11 ± 0.03^{a}	1.32 ± 0.01^{ab}	1.50 ± 0.05^{ab}	1.59 ± 0.02^{b}	1.21 ± 0.06^{ab}	1.23 ± 0.02^{ab}
GPx	108.71 ± 4.50^{a}	121.95 ± 8.29^{b}	140.23 ± 10.55^{c}	142.82 ± 12.47^{c}	123.64 ± 9.32^{b}	127.13 ± 10.90^{b}
GST	48.65 ± 3.72^{a}	54.07 ± 4.30^{b}	65.83 ± 4.04^{c}	65.08 ± 4.09^{c}	64.04 ± 4.49^{c}	58.69 ± 3.13^{b}
GR	34.88 ± 2.44^{a}	40.51 ± 4.05^{b}	41.71 ± 2.14^{b}	48.73 ± 1.54^{c}	54.27 ± 2.34^{d}	37.53 ± 1.69^{a}
GSH	5.10 ± 0.41^{a}	5.51 ± 0.47^{a}	8.16 ± 0.26^{d}	9.06 ± 0.28^{e}	7.19 ± 0.38^{c}	6.25 ± 0.24^{b}
VA contents	2.09 ± 0.18^{a}	2.35 ± 0.19^{b}	2.67 ± 0.11^{c}	2.66 ± 0.10^{c}	2.96 ± 0.10^{d}	3.10 ± 0.09^{d}
Regression						
$Y_{MDA\ in\ PI} = 2.845 \times 10^{-7} x^2 - 0.001x + 6.274$					$R^2 = 0.774$	P = 0.107
$Y_{MDA\ in\ MI} = 3.966 \times 10^{-7} x^2 - 0.002x + 4.279$					$R^2 = 0.802$	P = 0.088
$Y_{ROS\ in\ PI} = -1.483 \times 10^{-4} x + 0.916$					$R^2 = 0.909$	P < 0.01
$Y_{ROS\ in\ MI} = -2.723 \times 10^{-4} x + 0.952$; $Y_{min} = 0.521$					$R^2 = 0.933$	P < 0.05
$Y_{ASA\ in\ PI} = 0.024x + 57.950$; $Y_{max} = 85.075$					$R^2 = 0.988$	P = 0.069
$Y_{ASA\ in\ MI} = -5.577 \times 10^{-6} x^2 + 0.027x + 69.072$					$R^2 = 0.838$	P = 0.065
$Y_{ASA\ in\ DI} = -1.048 \times 10^{-5} x^2 + 0.046x + 75.284$					$R^2 = 0.964$	P < 0.01
$Y_{T\text{-}SOD\ in\ PI} = -4.507 \times 10^{-7} x^2 + 0.002x + 7.467$					$R^2 = 0.897$	P < 0.05
$Y_{T\text{-}SOD\ in\ MI} = -6.156 \times 10^{-7} x^2 + 0.002x + 8.709$					$R^2 = 0.771$	P = 0.109
$Y_{CuZnSOD\ in\ PI} = -4.176 \times 10^{-7} x^2 + 0.002x + 5.154$					$R^2 = 0.906$	P < 0.05
$Y_{CuZnSOD\ in\ MI} = -5.827 \times 10^{-7} x^2 + 0.003x + 5.369$					$R^2 = 0.791$	P = 0.096
$Y_{CAT\ in\ MI} = -1.257 \times 10^{-7} x^2 + 0.001x + 1.122$					$R^2 = 0.881$	P < 0.05
$Y_{GPx\ in\ PI} = 0.015x + 41.960$; $Y_{max} = 73.649$					$R^2 = 0.945$	P < 0.05
$Y_{GPx\ in\ MI} = -1.026 \times 10^{-5} x^2 + 0.044x + 38.742$					$R^2 = 0.804$	P = 0.087
$Y_{GST\ in\ PI} = -1.740 \times 10^{-6} x^2 + 0.008x + 42.786$					$R^2 = 0.850$	P = 0.058
$Y_{GST\ in\ DI} = -3.596 \times 10^{-6} x^2 + 0.016x + 47.940$					$R^2 = 0.915$	P < 0.05
$Y_{GR\ in\ PI} = -4.418 \times 10^{-6} x^2 + 0.020x + 20.211$					$R^2 = 0.974$	P < 0.01
$Y_{GR\ in\ MI} = -3.163 \times 10^{-6} x^2 + 0.016x + 23.313$					$R^2 = 0.909$	P < 0.05
$Y_{GR\ in\ DI} = -3.638 \times 10^{-6} x^2 + 0.016x + 32.503$					$R^2 = 0.739$	P = 0.133
$Y_{VA\ in\ the\ PI} = 7.640 \times 10^{-4} x + 4.030$; $Y_{max} = 5.536$					$R^2 = 0.942$	P < 0.05
$Y_{VA\ in\ the\ MI} = 3.424 \times 10^{-4} x + 2.420$; $Y_{max} = 3.136$					$R^2 = 0.995$	P < 0.01
$Y_{VA\ in\ the\ DI} = 2.994 \times 10^{-4} x + 2.160$; $Y_{max} = 3.033$					$R^2 = 0.932$	P < 0.01

Values are means ± SD. Except CF (n = 3*30), the n of the other indexes is six. Different superscript letters in the same row indicate significant differences between the treatments (P < 0.05).

CF, Condition factor (CF, %) = wet body weight × 100/(total body length)^{3}.

MDA (malondialdehyde, nmol/mg protein), PC (protein carbonyl, nmol/mg protein), AHR (anti-hydroxy radical, U/mg protein), ASA (anti-superoxide anion, U/g protein), T-SOD (total superoxide dismutase, U/mg protein), CuZnSOD (U/mg protein), MnSOD (U/mg protein), CAT (U/mg protein), GPx (U/mg protein), GST (U/mg protein), GR (U/g protein), GSH (glutathione, mg/g protein), VA (IU/kg).

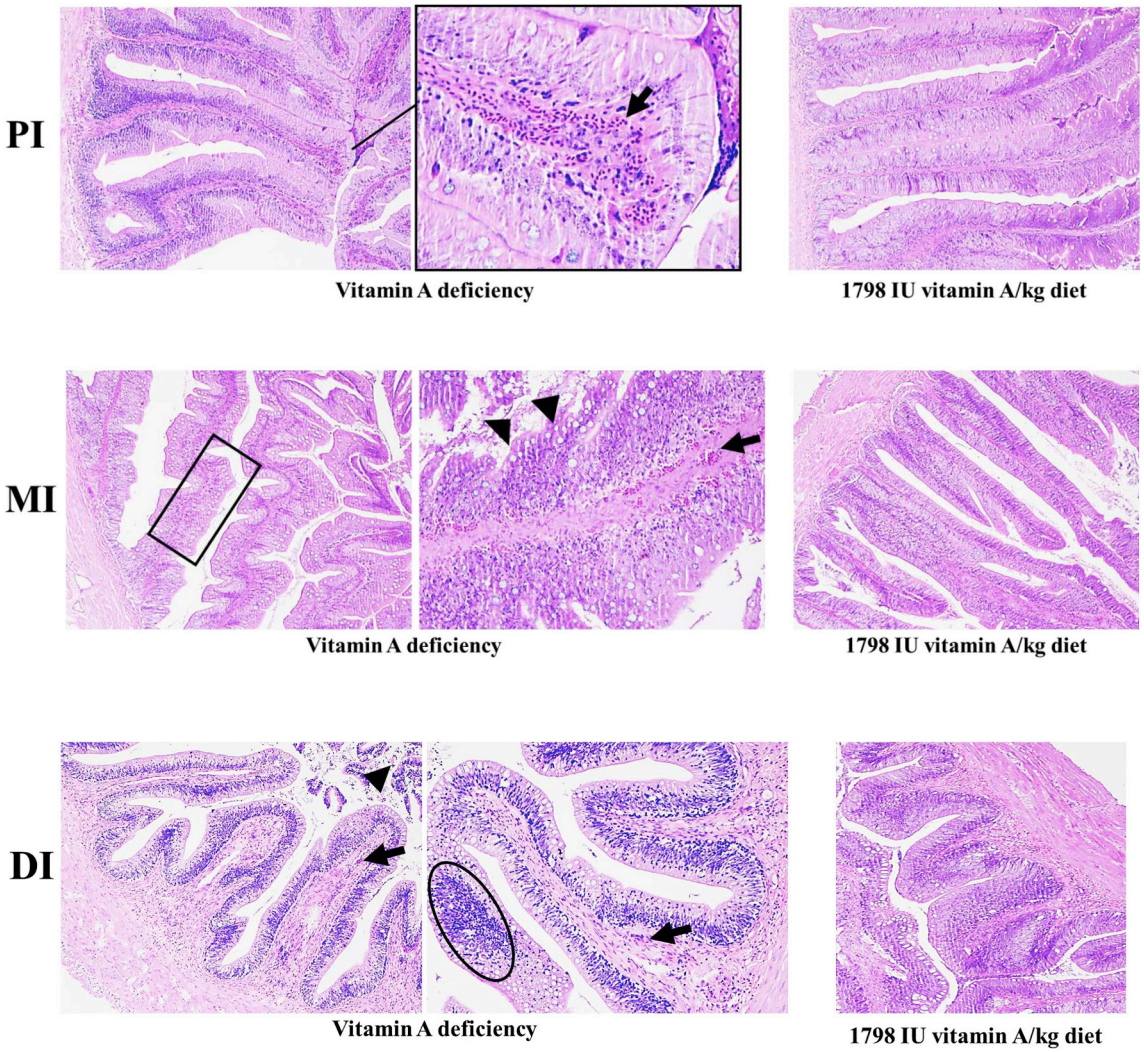

Fig. 1. Effects of VA on the intestinal histology in grass carp (*Ctenopharyngodon idella*). Arrowhead showed the blood capillary hyperaemia. Box showed a significant increase number of epithelial goblet cells. Trilateral showed the damage of the margin of intestinal villus. Ellipse showed the leucocyte infiltration. The sections were H & E staining and observed at 100 × original magnification.

diethylenetriaminepentaacetic acid (DETAPAC), 0.06 mM of nitro blue tetrazolium (NBT), 0.16 mM of xanthine solution and 30 μL of cell lysate. After the addition of 0.19 U/mL of xanthine oxidase, the absorbance change at 550 nm was monitored. To distinguish SOD isoforms, the sensitivity of CuZnSOD to cyanide was used because MnSOD is unaffected by this method. For CAT, the assay mixture consisted of 100 mM KPO_4 buffer (pH 7.0), 10 mM of H_2O_2 and 50 μL of cell lysate in a total volume of 1 mL. Decrease of H_2O_2 was monitored by measuring absorbance at 240 nm. For GPx, the reaction mixture consisted of tissue homogenates, 40 μL of 0.25 mM hydrogen peroxide (H_2O_2), 10 mM of sodium phosphate buffer (pH 7.0), 0.5 mM of GSH and 1.25 mM of NaN_3 in a total volume of 1 mL. After 3 min intervals, 0.5 mL of dithiobisnitrobenzoic acid was added. A yellow product formed as GSH reacts with dithiobisnitrobenzoic acid was monitored at 412 nm. GST activity was measured by monitoring the formation of the adduct between GSH and 1-chloro-2,4-dinitrobenzene (CDNB). For GSH, an adduct between GSH and CDNB was monitored at 340 nm. VA concentrations in each intestinal segment of the fish were determined with direct enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (ELISA, Cusabio Biotech, China) by the method of Serge Michel and Braham Shroot [43].

2.3. Real-time quantitative PCR

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR (RT-PCR) were close to our previous study [44]. In brief, the total RNA samples were isolated from the intestines using RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions, followed by DNAse I treatment. Then, cDNA was synthesized using the PrimeScript™RT reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions in a T100 thermal cycler (Bio-Rad Laboratories, USA). For quantitative real-time PCR, specific primers were designed according to the sequences cloned in our laboratory or the published sequences of grass carp (Table S2). All of the real-time PCR reactions were performed on a CFX96™Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.) using a SYBR® Prime Script RT-PCR Kit II. The stabilities of four commonly used internal control genes encoding *18S rRNA, β-actin, elongation factor 1 alpha (EF1-a),* and *glycer-aldehyde-3-phosphate dehydrogenase (GAPDH)* in the intestine of grass carp under the condition of the present study were assessed using the geNorm and NormFinder programs. 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 the cDNA loading. After verification that the primers amplified with an efficiency of approximately 100%, the $2^{-\Delta\Delta CT}$ method was used to calculate the mRNA levels of all the genes according to Livak and Schmittgen [45].

2.4. Statistical analysis

The results were shown as the mean ± standard deviation (SD). Data were transformed if necessary after evaluating assumptions of normality equality of variances and outliers, and were subjected to a one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range test to evaluate significant differences among treatments at $P < 0.05$ with SPSS 20.0 (SPSS Inc., Chicago, IL, USA), similar to the method of Li et al. [46]. Principal component analysis (PCA) was also performed. The scores of morphological changes were evaluated with the Student's *t*-test. Heat map diagrams showing VA-changed gene expression were created using Excel 2013 (Microsoft Corporation) software. The requirements of dietary VA based on intestinal health indicators were estimated by the quadratic regression model according to the method of Chen et al. [34].

3. Results

3.1. Growth performances and histopathological examination

This study used the same growth trial as our previous study which observed that an optimal VA could improve growth of grass carp (the final mean weight and SD of Group 1–6 are 836.09 ± 24.54[a], 888.01 ± 23.41[b], 939.54 ± 13.34[c], 996.67 ± 32.18[d], 906.17 ± 3.69[bc] and 848.89 ± 16.68[a] g, respectively) associated with the enhanced intestinal healthy by improvement of intestinal immune barrier function [9]. The condition factors of fish are presented in Table 1. Results showed that 1209–1798 IU/kg diet of VA increased the condition factors of fish compared to those in the control group and the highest levels of the VA group.

The histological results in fish PI, MI and DI are presented in Fig. 1. Compared with the optimal VA group, VA deficiency caused blood capillary hyperaemia in the PI, increased number of epithelial goblet cells, damage of the margin of intestinal villus and blood capillary hyperaemia in the MI and induced the damage of the margin of intestinal villus, blood capillary hyperaemia and the leucocyte infiltration in the DI of grass carp. A summary of the morphological changes observed is presented in Table 2. A general trend was noticed: compared with the optimal VA group, dietary VA deficiency could aggravate intestinal injuries in grass carp.

Table 2
Intestinal morphological changes in VA deficiency and optimal groups of grass carp[a].

	VA deficiency	1798 IU VA/kg diet
PI		
Blood capillary hyperemia	1.67	0.33
Significant increase number of epithelial goblet cells	0	0
Damage of the margin of intestinal villus	0	0
The leucocyte infiltration	0	0
Column totals	1.67[a]	0.33
MI		
Blood capillary hyperemia	1.33	0
Significant increase number of epithelial goblet cells	2.33	0.33
Damage of the margin of intestinal villus	1.67	0.33
The leucocyte infiltration	0	0
Column totals	5.33[a]	0.66
DI		
Blood capillary hyperemia	1.33	0.33
Significant increase number of epithelial goblet cells	0	0
Damage of the margin of intestinal villus	1.33	0
The leucocyte infiltration	2.33	0
Column totals	4.99[a]	0.33

[a] The morphological changes are based on light microscopy evaluation of 10 micrographs from each fish in each treatment group. Tissue changes were assessed as follows: 0 = not observed; 1 = low frequency (1–3 out of 10 images); 2 = moderate frequency (4–6 out of 10 images) and 3 = high frequency (7 or more out of 10 images); Means, N = 3 fish. Star in the same row means significant difference.

3.2. Oxidation and antioxidant-related parameters in three intestinal segments of the fish

Oxidation and antioxidant-related parameters in the PI, MI and DI of fish are presented in Fig. 2 and Table 1. In the PI, ROS production was significantly decreased with increasing VA levels up to 3796 IU/kg diet ($P < 0.05$). MDA and PC contents in the PI of grass carp were gradually decreased with VA levels up to 1209–1798 IU/kg diet and 1798 IU/kg diet ($P < 0.05$), respectively, and then gradually increased. The ASA and GPx activities as well as VA content in the PI was increased with dietary VA levels up to 1209, 1798 and 1798 IU/kg ($P < 0.05$), respectively, and plateaued thereafter ($P > 0.05$). The T-SOD, CuZnSOD, CAT, GST and GR activities as well as the GSH content in the PI of grass carp were gradually increased with VA levels up to 1209–1798 IU/kg diets, and then gradually decreased. In the MI, ROS content was significantly decreased with dietary VA levels up to 1798 IU/kg ($P < 0.05$), and reached a plateau thereafter ($P > 0.05$). MDA and PC contents in the MI of grass carp were significantly decreased with VA levels up to 1209 IU/kg diet and 606.8 IU/kg diet ($P < 0.05$), respectively, and then gradually increased. The ASA, T-SOD, CuZnSOD, CAT, GPx, GST and GR activities and the GSH content in the MI of grass carp were gradually increased with dietary VA levels up to 1798, 1209, 1209, 1798–2805, 1209–1798, 1209–1798, 2805 and 1798 IU/kg ($P < 0.05$), respectively, and then gradually decreased. VA content in the MI was significantly improved with dietary VA levels up to 1798 IU/kg ($P < 0.05$) and plateaued thereafter ($P > 0.05$). In the DI, the ROS, MDA and PC contents were significantly decreased with dietary VA levels up to 1798, 1209–1798 and 606.8–1798 IU/kg ($P < 0.05$), respectively, and beyond the levels, the contents significantly improved ($P < 0.05$). The ASA, T-SOD, CuZnSOD, CAT, GPx, GST and GR activities as well as the GSH contents in the DI were gradually or significantly increased with dietary VA levels up to 1798, 1209, 1209, 1798, 1209–1798, 1209–2805, 1798 and 1798 IU/kg, respectively, and then gradually decreased thereafter. VA content in the DI was significantly increased with dietary VA levels up to 2805 IU/kg ($P < 0.05$) and plateaued thereafter ($P > 0.05$). However, dietary VA did not significantly alter the AHR and MnSOD activities in three intestinal segments of the fish ($P > 0.05$).

As shown in Fig. 3, in the PI, the *CuZnSOD*, *CAT, GPx1a*, *GPx4a*, *GSTr,* and *GR* mRNA levels were all gradually up-regulated with dietary VA levels up to 1798 IU/kg, and then gradually down-regulated with higher VA levels. The *Nrf2* mRNA in the PI was gradually up-regulated with dietary VA levels up to 606.8 IU/kg, and then gradually down-regulated. The *Kelch-like ECH-associating protein (Keap1a)* mRNA level in the PI was significantly down-regulated with dietary VA levels up to 1209 IU/kg ($P < 0.05$) and plateaued thereafter ($P > 0.05$). However, VA had no significant effects on the *MnSOD, GPx1b, GPx4b, GSTp1, GSTp2, GSTo1, GSTo2* and *Keap1b* mRNA levels in the PI ($P > 0.05$). The *CuZnSOD*, *CAT, GPx1a, GPx4a, GSTr, GSTp1, GR* and *Nrf2* mRNA levels in the MI were gradually up-regulated with dietary VA levels up to 1209, 1798, 1798, 1798, 1798, 1798, 1798 and 1209 IU/kg, respectively, and gradually down-regulated with higher VA levels. The *GSTo1* mRNA level in the MI was gradually up-regulated with dietary VA levels up to 1798 IU/kg and plateaued thereafter. The Keap1a mRNA level in the MI was gradually down-regulated with dietary VA levels up to 1798 IU/kg, and beyond the level, the mRNA was

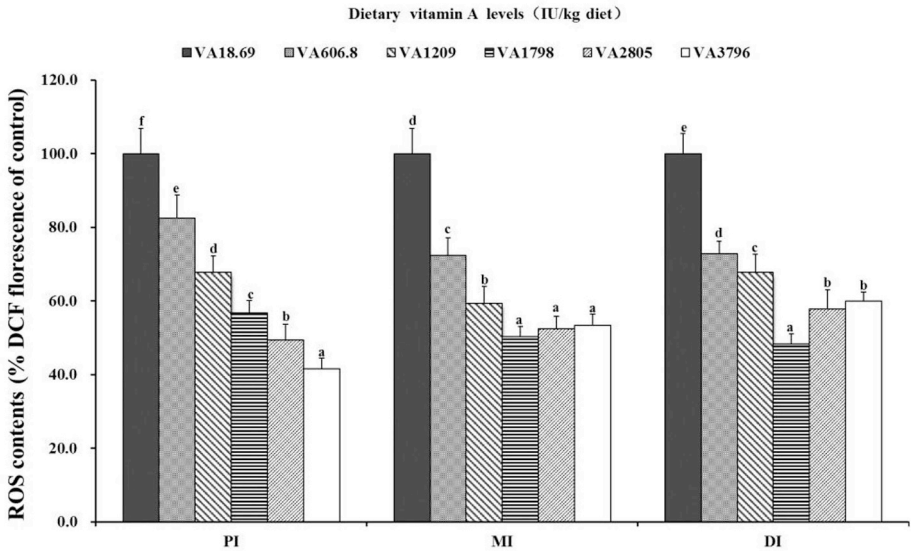

Fig. 2. Effects of dietary VA on reactive oxygen species (ROS) contents in the PI, MI and DI of grass carp. Values are means (n = 6), error bars indicate S.D. Different letters indicate the significant difference ($P < 0.05$).

significantly improved ($P < 0.05$). However, VA had no significant effects on the *MnSOD, GPx1b, GPx4b, GSTp2, GSTo2* and *Keap1b* mRNA levels in the MI ($P > 0.05$). The *CuZnSOD*, *CAT, GPx1a, GPx4a, GPx4b*, *GSTo1, GSTr*, *GSTp1, GR* and *Nrf2* mRNA levels in the DI were gradually up-regulated with dietary VA levels up to 1798, 1798, 1798, 1209, 1209, 1209, 1798, 1798, 2805 and 1798 IU/kg, respectively, and gradually down-regulated thereafter. Fish fed the VA-deficient diet and 3796 IU/kg diet showed higher Keap1a mRNA levels than other groups in the DI ($P < 0.05$). However, the *MnSOD, GPx1b, GSTp2, GSTo2* and *Keap1b* mRNA levels in the DI were not all significantly affected by dietary VA levels ($P > 0.05$).

3.3. Apoptosis-related parameters and TOR signalling-related factors in three intestinal segments of fish

As shown in Fig. 3, in the PI, the *caspase-3, caspase-8, caspase-9*, apoptotic protease *activating factor-1 (Apaf-1), Fas ligand (FasL)*, *eIF4E-binding protein 1 (4E-BP1)* and *B-cell lymphoma protein 2 associated X protein (Bax)*mRNA levels were gradually down-regulated with the VA levels up to 1798, 1209, 1209, 1798, 1798, 1798 and 1798 IU/kg diet, respectively, and then gradually up-regulated. The *B-cell leukaemia/lymphoma-2 (Bcl-2), S6 kinases1 (S6K1)* and *TOR* mRNA levels in the PI were gradually up-regulated with VA levels up to 1209, 1209 and 1798 IU/kg diet, respectively, and gradually down-regulated with higher VA levels. In the MI, the mRNA levels of *caspase-3, caspase-8, caspase-9, FasL* and *Bax* were all gradually down-regulated with dietary VA levels up to 1798 IU/kg and then gradually improved. The mRNA levels of *Apaf-1* and *4E-BP1* in the MI were all significantly down-regulated with dietary VA levels up to 1209 IU/kg ($P < 0.05$), and then gradually improved. The *Bcl-2* and *S6K1* mRNA levels in the MI were gradually up-regulated with dietary VA levels up to 1209 IU/kg and 1798 IU/kg, respectively, and then gradually down-regulated with higher VA levels. The *TOR* mRNA level in the MI was lower for fish fed VA-deficient diet than the VA-supplemented diet ($P < 0.05$), and there were no significant differences among these VA-supplemented groups ($P > 0.05$). In the DI, the *caspase-3, caspase-8, Bax, FasL* and *caspase*-9 mRNA levels were gradually down-regulated with dietary VA levels up to 1798, 1209, 1209, 1209 and 1209 IU/kg, respectively, and gradually up-regulated thereafter. The *Apaf-1* and *4E-BP1* mRNA level in the DI was down-regulated with dietary VA levels up to 1209 and 1798 IU/kg, respectively, and plateaued with higher VA levels. The *TOR, Bcl-2* and *S6K1* mRNA levels in the DI were all gradually up-regulated with dietary VA levels up to 1798 IU/kg and then gradually down-regulated with higher VA levels. However, dietary VA had no significant effects on *caspase-7* mRNA level in three intestinal segments of the fish ($P > 0.05$).

3.4. TJ complexes and MLCK mRNA levels in three intestinal segments of the fish

The effect of VA on the related TJ complexes and *MLCK* mRNA levels are showed in Fig. 3. In the PI, the mRNA levels of *claudin-b, claudin-c, claudin-3, claudin-12* and *occludin* were gradually up-regulated with dietary VA levels up to 1798, 1798, 1798, 1209 and 1209 IU/kg, respectively, and then gradually down-regulated. Fish fed the VA-deficient diet showed lower *claudin-15a* mRNA level than the VA-supplemented groups in the PI ($P < 0.05$), and no differences occurred among these VA-supplemented groups ($P > 0.05$). The *ZO*-1 mRNA in the PI was gradually up-regulated with dietary VA levels up to 1209 IU/kg, and plateaued with higher VA levels. The *MLCK* mRNA level in the PI was gradually down-regulated with the VA levels up to 1798 IU/kg, and then gradually up-regulated. However, the *claudin-7* and *claudin-11a* mRNA levels were all not affected by VA levels in the PI. In the MI, the mRNA levels of *claudin-b, claudin-c, claudin-3, claudin-11a, claudin-15a* and *ZO-1* were all gradually up-regulated with dietary VA levels up to 1798 IU/kg, and gradually down-regulated thereafter. The *claudin*-12 mRNA level in the MI was gradually up-regulated with dietary VA levels up to 1209 IU/kg, and gradually down-regulated thereafter. The *claudin*-7 mRNA level in the MI was gradually up-regulated with dietary VA levels up to 1798 IU/kg, and plateaued with higher VA levels. Fish fed the VA-deficient diet showed significant lower *occludin* mRNA level than those fed VA-supplemented diets in the MI ($P < 0.05$), and there were no significant differences among these VA-supplemented groups ($P > 0.05$). The *MLCK* mRNA level in the MI was gradually down-regulated with dietary VA levels up to 1209 IU/kg, and gradually improved thereafter. In the DI, the mRNA levels of *claudin-b, claudin-c, claudin-3, claudin-7, claudin-12* and *ZO-1* were all gradually up-regulated with dietary VA levels up to 1798 IU/kg, and gradually down-

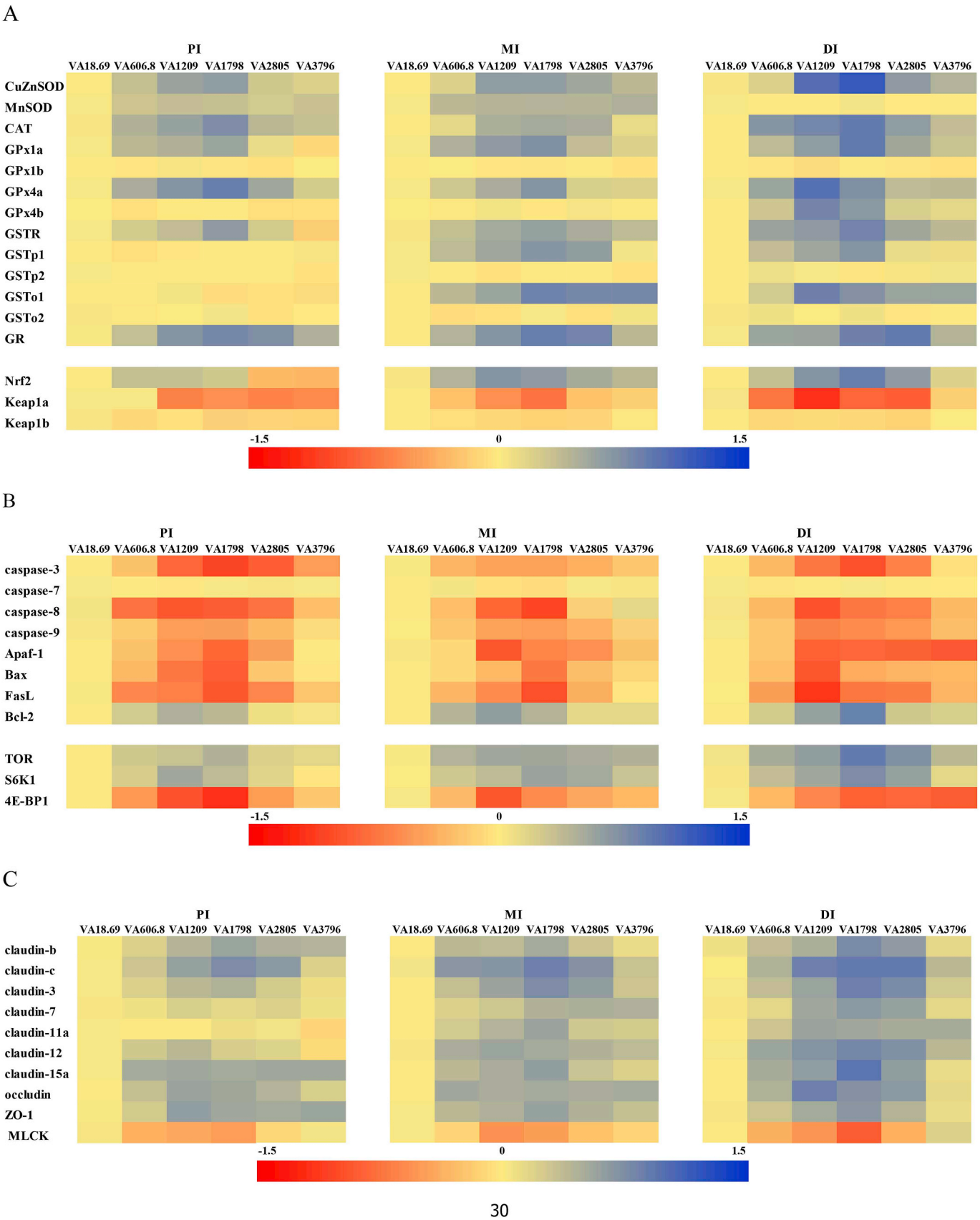

Fig. 3. Heat-map of VA-changed expression of selected genes involved in antioxidant (A), apoptosis (B) and TJ (C) in the PI, MI and DI of fish. The signal values of up-regulation (blue) and down-regulation (red) were expressed as Log_2Fold and ranged from 1.5 to −1.5 folds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

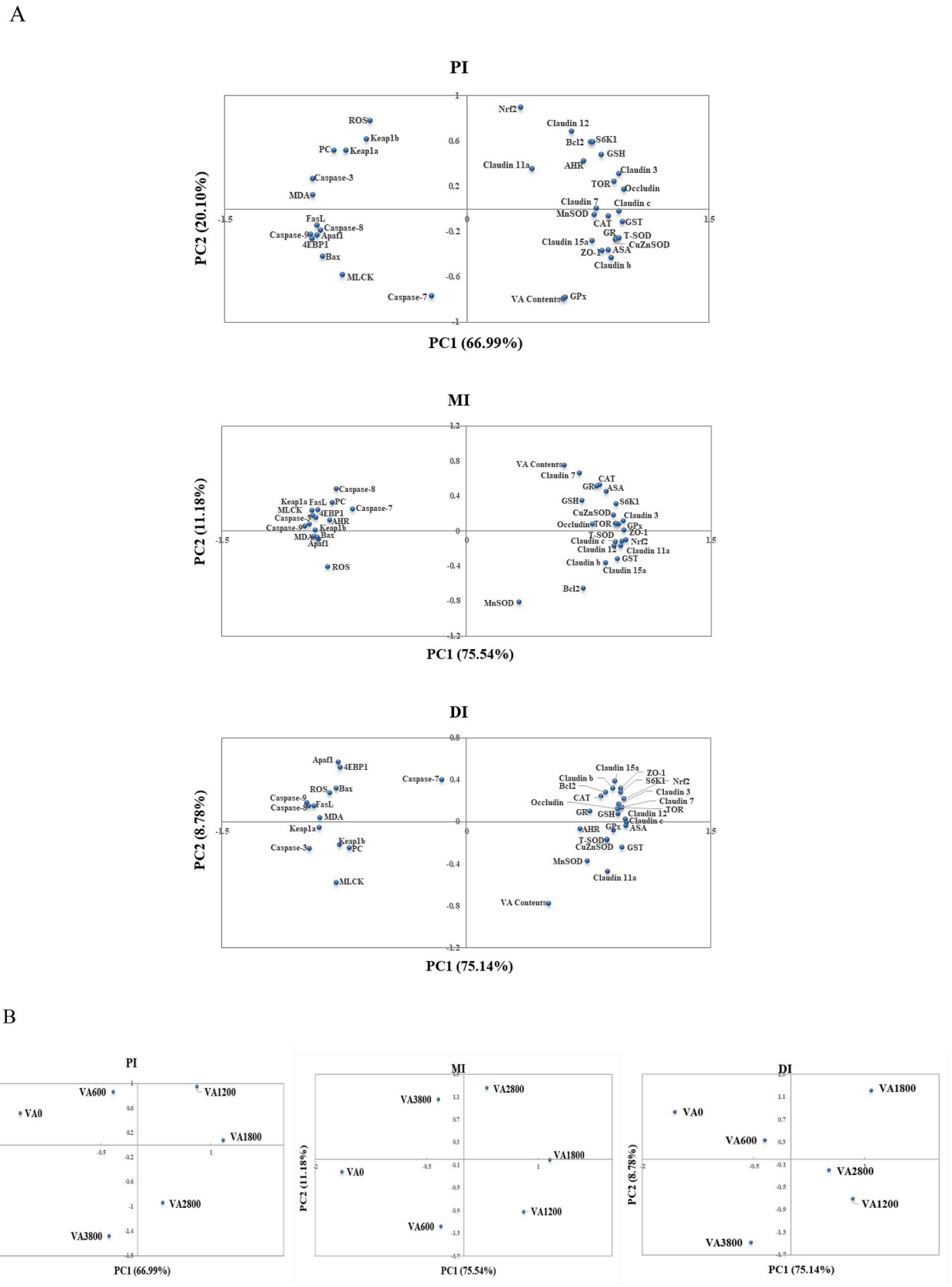

Fig. 4. PCA loading plot (Fig. 4A) and score plot (Fig. 4B, mean values) from oxidative, antioxidant, apoptosis and TJ parameters showing the first two principal components (PC1 and PC2).

regulated thereafter. The *claudin-15a* and *occludin* mRNA level in the DI was up-regulated with dietary VA levels up to 1798 and 1209 IU/kg, respectively, and gradually down-regulated with higher VA levels. The *claudin-11a* mRNA level in the DI was gradually up-regulated with dietary VA levels up to 1209 IU/kg and plateaued thereafter. The *MLCK* mRNA level in the DI was gradually down-regulated with dietary VA levels up to 1798 IU/kg, and gradually up-regulated with higher VA levels.

3.5. PCA

The PCA results are given in Fig. 4. It was shown that approximately 87.09%, 86.72% and 83.92% of the variability in the PI, MI and DI, respectively, were explained by first two principal components. Generally, principal component 1 (PC1) in the PI (66.99%), MI (75.54%) and DI (75.14%) were all positively related to antioxidants (Nrf2, and activities of T-SOD, CuZnSOD, MnSOD, CAT, GST, GR, and VA contents) and TJs (Occludin, Claudins and ZO-1), and were all negatively related to oxidative damage and apoptosis parameters. The PCA score plot shows that VA deficiency (VA0 and VA600 groups) and excess (VA3800 group) in the PI, MI and DI were all in the left part of the graph, suggesting that poor intestinal structural integrity in these three groups compared with those of other groups.

4. Discussion

Our previous study observed that an optimal VA could improve growth of grass carp associated with enhanced intestinal health by improvement of intestinal immune barrier function [9]. In addition to immune barrier function, intestinal physical barrier function also plays important roles in the intestinal health of animals. Thus, this study was the first to investigate the effects of VA on the intestinal physical barrier of fish. Studies have shown that the excessive increase of epithelial goblet cells in rats [47], as well as blood capillary hyperaemia and the leucocyte infiltration in juvenile Jian carp [48] are pathological symptoms. In this study, histologic results showed that compared with optimal VA, dietary VA deficiency led to the obvious and statistical significance symptoms of damage of the margin of intestinal villus, blood capillary hyperaemia, leucocyte infiltration and excessive increase of epithelial goblet cells in the intestines of fish, suggesting that VA deficiency damaged the intestinal physical barrier function of fish. Damage of the intestinal physical barrier function is always related to the impaired cellular and cell-cellular structural integrity in fish [49], which is our next topic of focus.

4.1. VA deficiency impaired cellular and cell-to-cell structural integrity linked to Nrf2, TOR and MLCK signalling in the intestine of fish

Cellular oxidative damage could impair the integrity of intestinal epithelia of fish [50]. High concentrations of ROS have been shown to produce adverse modifications to cell components [51], which could be scavenged by enzymatic and non-enzymatic antioxidants in fish [52]. The current study observed that, compared with optimal VA levels, VA deficiency increased the ROS, MDA and PC contents, and decreased ASA, CuZnSOD, CAT, GPx, GST and GR activities, as well as GSH and VA contents in three intestinal segments of young grass carp, suggesting that VA deficiency caused oxidative damage partly related to the decreased antioxidant ability in the intestines of fish. Recent study in our lab showed that antioxidant enzymes such as CuZnSOD and GR activities were positively related to their respective mRNA levels in fish [53]. We further observed that VA deficiency down-regulated most of the studied antioxidant enzyme mRNA levels in three intestinal segments, and these antioxidant enzyme activities were positively correlated with their corresponding gene expressions (Table S3), suggesting that VA deficiency decreased antioxidants enzyme activities may be partly related to the down-regulation of their corresponding gene transcriptions in the fish intestine. It should be noted that the mRNA levels of *GPx1b*, *GSTp2* and *GSTo2* were not affected by VA levels in three intestinal segments, suggesting that GPx activity decreased by VA deficiency may be not occur through *GPx1b* mRNA levels, while decreased GST activity might be independent of *GSTp2* and *GSTo2* mRNA levels in the fish intestines. To protect young grass carp against the PC, the optimum VA level was estimated to be 2622 IU/kg diet (Fig. S1).

Meanwhile, we found three interesting results. The first is that compared with optimal VA, VA deficiency only decreased ASA activity (rather than AHR activities) in the intestine of fish, which might be partially because of its conjugated diene structure characteristics. ASA activity and AHR activity are two indexes used to evaluate $O_2^{\bullet -}$ scavenging ability and •OH-scavenging ability, respectively [51]. VA was proven to be an effective inhibitor of superoxide radical ($O_2^{\bullet -}$) production induced by human polymorphonuclear leukocytes [54]. However, the mechanisms need to be further investigated. The second interesting result is that VA only decreased the activities and mRNA levels of CuZnSOD (rather than MnSOD) in the intestine of fish. The unchanged MnSOD activities might be partially associated with the MnSOD gene transcriptional level. To our knowledge, the antioxidant enzyme activities were in part a consequence of the antioxidant enzyme gene transcription in rats [55]. This study observed that the mRNA levels of *MnSOD* were not affected by VA, supporting our hypothesis. The different effects of VA on intestinal *CuZnSOD* and *MnSOD* mRNA levels in fish might be associated in part with their cellular localisation. CuZnSOD is localised to cytoplasm, whereas the MnSOD is found in the matrix of mitochondria in fish [56]. To our knowledge, the cytoplasm is the place where VA metabolised to its major active metabolite retinoic acid (RA) in fish [57]. However, this hypothesis requires further investigation. The third interesting result is that VA did not change most GST isoforms in the PI (rather than MI and DI), which may be partly related to the similar function of VA and GST in antioxidants. GSTs represent an important group of enzymes that could detoxify both endogenous compounds and foreign chemicals [65], whereas VA could also play an important role in detoxifying oxygen radicals in rats [66]. This study observed that VA content in the PI was much higher than that in the MI and DI, supporting our hypothesis.

The down-regulation of most of the antioxidant enzyme mRNA levels by VA deficiency might be partly linked to Nrf2 signalling. In mice, Nrf2 plays a pivotal role in antioxidant enzyme gene expression [58], which can be bound and inhibited by Keap1 (including Keap1a and Keap1b) in zebrafish [59]. In the present study, compared with optimal VA, VA deficiency down-regulated Nrf2 mRNA levels, and only up-regulated Keap1a (rather than Keap1b) mRNA levels in three intestinal segments of fish. Further correlation analysis indicated that *CuZnSOD*, *CAT*, *GPx4a*, *GSTr, GSTp1* and *GR* mRNA levels in the MI and DI, *GPx1a* mRNA level in the PI, MI and DI as well as *GPx4b* mRNA level in the DI were positively correlated with *Nrf2* mRNA levels of the fish (Table S3), supporting that these antioxidant enzyme mRNA levels decreased by VA deficiency might be partly ascribed to *Keap1a* (not *Keap1b*)/Nrf2 signalling. The reason for the different effects of VA on *Keap1a* and *Keap1b* mRNA levels may be associated with phospholipids (PL). VA has been reported to enhance surfactant PL synthesis in rats [60]. Our previous study observed that PL could also only up-regulate *Keap1a* (but not *Keap1b*) expression in the fish intestine [53]. However, the underlying mechanisms warrant further investigation.

Oxidative damage can lead to cellular apoptosis. Apoptosis is essential for the removal of neutrophils from inflamed tissues and the timely resolution of inflammation. However, excessive apoptosis could destroy the cellular structural integrity of the fish intestine [61].

Cellular apoptosis was performed mainly through the external death receptor (FasL/caspase-8) and the internal mitochondrial pathway [(Bcl-2, Mcl-1 and Bax)/caspase-9] in rat hepatocytes [62]. Caspase-3 and caspase-7 were the two main downstream effector caspases which were directly responsible for the cellular component degradation in fish [63]. In this study, compared with optimal VA, VA deficiency only up-regulated *caspase-3* (but not *caspase-7*), *caspase-8* and *caspase-9*, as well as pro-apoptotic members *FasL*, *Bax* and *Apaf*-1 mRNA, while down-regulated anti-apoptotic protein *Bcl*-2 mRNA levels in three intestinal segments of fish. Correlation analysis indicated that *caspase*-3 mRNA was positively correlated with *caspase-8* and *caspase*-9 mRNA levels, while *caspase-8* and mRNA level was positively correlated with *FasL* mRNA level, and *caspase*-9 mRNA was positively correlated with *Bax* and *Apaf*-1 mRNA levels and negatively correlated with *Bcl*-2 mRNA level (Table S3). Furthermore, it was reported that inhibiting TOR/S6K1 [64] and enhancing 4E-BP1 [65] could induce apoptosis in human tumour cells. In the present study, compared with the optimum VA levels, dietary VA deficiency down-regulated *TOR* and *S6K1* mRNA levels and up-regulated *4E-BP1* mRNA levels in three intestinal segments of the fish. These results corroborate the idea that the VA deficiency could aggravate apoptosis partly related to the inhibition of the TOR signalling pathway to regulate the mitochondria pathway [(Bcl-2, Mcl-1 and Bax)/Apaf-1/caspase-9/caspase-3 (rather than caspase-7)] and the death receptor pathway [FasL/caspase-8/caspase-3, -7 (rather than caspase-7) in the intestines of fish. The reason for the negligible effects of VA on *caspase-7* expression might be in part associated with the adhesion effect of VA. VA has been reported to up-regulate the expression of cell adhesion-related proteins to enhance the normal cell–cell adhesion in rats [66]. Evidence has shown that caspase-3 could inhibit cell adhesion more rapidly than caspase-7 in mice [67]. However, this hypothesis requires further investigation.

In general, the induction of cell apoptosis and the damage of the antioxidant capacity in part contribute to the damage of fish cellular integrity. In addition to the intestinal cellular integrity, fish intestinal structural integrity also relies on integrity of the cell-to-cell TJ complexes [14], which could be disrupted by the activation of MLCK in rats [68]. The present study observed that compared with the optimum VA levels, dietary VA deficiency down-regulated the *claudin-b*, *claudin-c*, *claudin-3*, *claudin-12*, *claudin-15a*, *occludin* and *ZO*-1 mRNA levels in the PI, MI and DI as well as *claudin-7* and *claudin-11a* mRNA levels in the MI and DI, and up-regulated *MLCK* mRNA levels in three intestinal segments of fish. Correlation analysis showed that *claudin-b, claudin-3*, *claudin-12*, *claudin-15a*, *occludin* and *ZO*-1 mRNA levels were negatively related to *MLCK* expression (Table S3), indicating that the decrease of TJ gene expression by VA deficiency might be partly related to the increase of *MLCK* expression in the fish intestine.

Interestingly, the effects of VA on TJ complexes in the PI were different than those of the MI and DI of the fish. In this study, compared with the appropriate VA levels, dietary VA deficiency down-regulated the mRNA levels of *claudin-7* and *claudin-11a* in the MI and DI (rather than in the PI). This may be due in part to the regulation of nuclear factor (NF)-κB and p38MAPK signalling. A previous study reported that NF-κB could up-regulate *claudin*-7 mRNA expression in XS52 cells [69], while the activation of p38MAPK could lead to down-regulation of *claudin*-11 mRNA in 15P-1 Sertoli cells [70]. Our previous study observed that both *NF-κB p65* and *p38MAPK* in the PI were not affected by VA levels [9], supporting our hypothesis.

4.2. Excessive VA damaged fish physical barrier functions in the PI, MI and DI of fish

The present study also showed that excessive VA increased most of the pro-apoptotic-related parameters, decreased most of the anti-oxidant-related parameters and down-regulated most of the TJ complex mRNA levels in the PI, MI and DI of grass carp, indicating that excessive VA impaired the physical barrier in three intestinal segments of the fish. The negative effects of excessive VA on fish intestinal physical barrier functions may be partly associated with the unbound free retinol. It is well known that VA is normally transported in plasma as retinol bound to a specific protein, retinol-binding protein (RBP), to target tissues [71]. Meanwhile, retinol-binding protein type II (CRBPII), which could bind retinol at the cellular level, is present at high concentrations in the enterocytes [72]. Previous study have reported that excessive doses of VA resulted in a significant decrease of serum RBP levels [73] and caused the limitation in the binding capacity of CRBP (II) in rats [74]. Therefore, excessive VA might induce an increase of the unbound free retinol. To our knowledge, excessive unbound free retinol is toxic to biological membranes through its detergent property in animals [74,75]. Thus, excessive VA showed an impairment of physical barrier functions which might be ascribed to damage to the intestine caused by unbound free retinol. However, the underlying mechanism requires further investigation.

4.3. PCA analysis

PCA loading plot showed that the TJs positively correlated with the antioxidant index, and negatively correlated with the oxidative damage and apoptosis parameters in the intestines of fish, especially in the MI and DI, suggesting that higher antioxidant ability might be a benefit for the TJs, but oxidative damage and apoptosis may be related to the disruption of the TJs. PCA score plot showed the separation of VA treatment groups. The middle levels of VA (VA1200, 1800 and 2800) were located in the positive part of PC1, and were separated from the low (VA0 and VA600) or high (VA3800) levels of VA in the PI, MI and DI of grass carp. Meanwhile, the VA1200 and 1800 groups in the PI, VA2800 in the MI and VA1800 in the DI were located in the positive part of both PC1 and PC2, indicated that those doses in corresponding intestinal segments might be contributed greatly to the structural integrity of the intestines of fish.

5. Conclusion

Summary above (Fig. 5), this study was the first to observe that VA deficiency might disrupt fish intestinal physical barrier function referring to cause oxidative damage, cell apoptosis and TJ complex disruption. Briefly, the PI, MI and DI, VA deficiency: (1) caused the oxidative damage of intestines partly related to decrease of antioxidant capacity by decreasing the activities and mRNA levels of *CuZnSOD* (except *MnSOD*), *CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTp1, GSTo1* (not *GSTp2* and *GSTo2*) and *GR*, which might be associated with the inhibition of the *Nrf2/Keap1a* (not *Keap1b*) signalling pathway; (2) promoted cell apoptosis indicated by up-regulation of *caspase-3, -8* and *-9* (rather than *caspare-7*) mRNA levels, which might be partly related to the inhibition of TOR signalling to up-regulate *FasL* and pro-apoptosis proteins (*apaf-1, bax*) as well as down-regulate the anti-apoptosis protein *Bcl*-2 mRNA levels; (3) impaired fish intestinal cell-to-cell integrity indicated by up-regulation of MLCK signalling to down-regulation of *claudin-b, claudin-c, claudin-3, claudin-12, claudin-15a, occludin* and *ZO*-1 mRNA levels. Different intestinal segments showed different responses to VA deficiency. In the MI and DI, VA deficiency could down-regulate *GSTp2*, *GSTo2*, *claudin-7* and *claudin-11a* mRNA levels, whereas in the PI, these genes were not affected by VA levels. Meanwhile, excessive VA also showed similar negative effects on fish intestinal barrier functions. Finally, based on protecting fish against the PC, the optimum VA levels for young grass carp were estimated to be 2622 IU/kg diet.

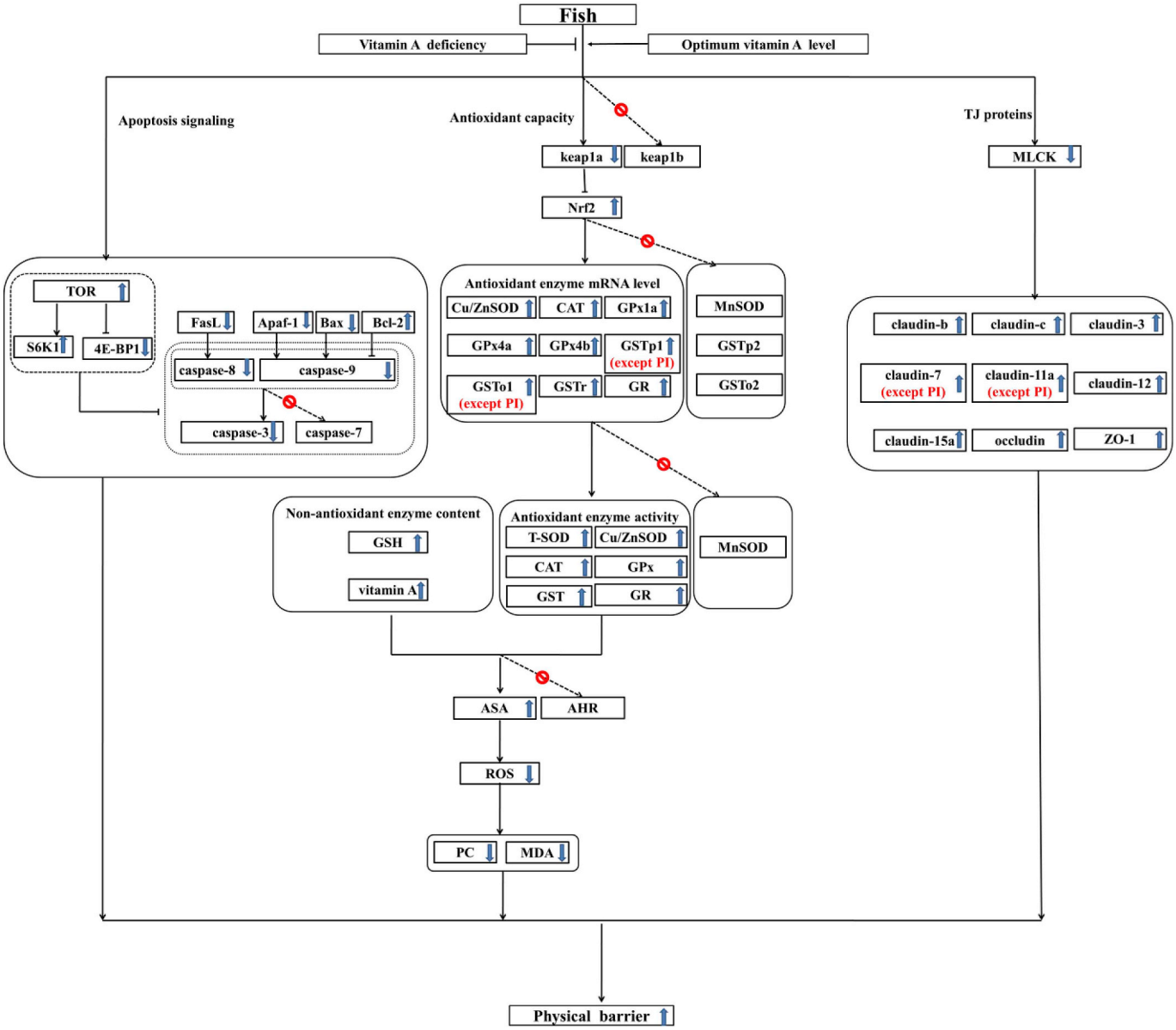

Fig. 5. General summary for the effects of VA on physical barrier function and its potential signalling pathways in the intestines of fish.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.056.

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