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Dietary conjugated linoleic acid modulates morphology, selective immune parameters, and gene expressions in the intestine of grass carp

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ABSTRACT

Conjugated linoleic acid (CLA) has been shown to exhibit anti-inflammatory properties in the intestine in mammals. However, the effect of CLA on intestinal immune response in fish is still unknown. Therefore, a 65-day growth trial was conducted to investigate the effects of dietary conjugated linoleic acid (CLA) on morphology, selective immune parameters, and gene expressions in the intestine of grass carp. Seven isonitrogenous and isolipidic diets were formulated as follows: 0 (control), 0.5 (CLA0.5), 1 (CLA1), 1.5 (CLA1.5), 2 (CLA2), 2.5 (CLA2.5), and 3 (CLA3) g CLA per 100g of feed.

Results: showed that dietary supplementation of 1.5–3% CLA significantly ($P < 0.05$) increased the fold and enterocyte heights in the PI and MI of grass carp. Complement 3 (C3) and immunoglobulin M (IgM) contents in three intestinal segments were significantly ($P < 0.05$) higher in fish fed with CLA1.5 to CLA2.5 diets compared to fish fed the control diet. CLA1.5 to CLA2.5 diets significantly ($P < 0.05$) increased the mRNA expression levels of anti-inflammatory cytokines (IL-10 and TGFβ1) and significantly ($P < 0.05$) reduced the mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-8, and TNF-α) in the PI, MI, and DI. This improved expression of anti-inflammatory cytokines and the inhibited expression of pro-inflammatory cytokines in the intestine of grass carp, might be mediated via TLR4/NF-κB-signaling pathway. Our results suggested that CLA1.5 to CLA2 diets improved intestinal morphology, increased the expression of anti-inflammatory cytokines, and inhibited the expression of pro-inflammatory cytokines in the intestine of grass carp. In conclusion, dietary supplementation of 1.5%–2% CLA show the anti-inflammatory therapeutic potential in the intestine of grass carp. The anti-inflammatory therapeutic potential of CLA might be mediated via TLR4/NF-κB-signaling pathway.

1. Introduction

The intestine is an important organ for nutrient digestion and absorption in fish and it plays an important role in the prevention of invasion by antigens and pathogens [1]. Sundh [2] observed that the impaired intestinal barrier function increases disease susceptibility in Atlantic salmon (*Salmo salar* L.). In this regard, the intestinal barrier function is important to maintain health of fish. Studies conducted recently have shown that several vitamins improved the intestinal barrier in grass carp [3–7]. Clearly, dietary supplementation with nutrients are considered as the major strategies to increase the intestinal barrier function in fish [8,9].

Inflammation is one of the most important immune responses, which majorly mediated by cytokines and inflammation can also

Abbreviations: CLA, conjugated linoleic acid; DI, Distal intestine; IFNγ2, interferon gamma 2; IκBα, NF-kappa-B inhibitor subunit alpha; IKKα, inhibitor of nuclear factor kappa-B kinase subunit alpha; IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta; IKKγ, inhibitor of nuclear factor kappa-B kinase subunit gamma; IL-1β, interleukin-1beta; IL-8, interleukin 8; IL-10, interleukin 10; IRAK-1, interleukin-1 receptor-associated kinase 1; MI, Mid intestine; MyD88, Myeloid differentiation primary response protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NF-κB p65, nuclear factor kappa-light-chain-enhancer of activated B cells p65 subunit; PI, Proximal intestine; TAK1, Transforming growth factor beta-activated kinase 1; TGFβ1, transforming growth factor beta 1; TLR4, toll-like receptor 4; TNFα, Tumor necrosis factor alpha; TRAF6, TNF receptor-associated factor 6

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activate and modulate the immune response [10]. Tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) are crucial pro-inflammatory cytokines and they are able to increase the synthesis of small inflammatory mediators. Meanwhile, transforming growth factor β (TGF-β) and interleukin 10 (IL-10) appear to control the production of inflammatory cytokines to limit inflammatory responses [11]. Conjugated linoleic acid (CLA) is a mixture of positional and geometrical isomers of linoleic acid [12]. CLA exhibits anti-inflammatory properties through the inhibition of cyclo-oxygenase-2 (COX-2) via decreasing the release of prostaglandin E_2 (PGE2) and downstream pro-inflammatory cytokine production [13,14] both *in vivo* and *in vitro* studies. Moreover, CLA feeding has beneficial effects on inflammatory- and endotoxin-induced anorexia and mucosal damage in experimental colitis [15,16]. Furthermore, CLA-activated PPARγ in cell lines binds to and inhibits nuclear factor-κB (NF-κB) activation and translocations into the nucleus in a similar manner as the inhibitor of NF-kappa-B inhibitor (IκB) [17,18]. Thus, this receptor reduces NF-κB-activated pro-inflammatory cytokine formation [19]. Therefore, CLA shows the anti-inflammatory therapeutic potential in inflammatory-mediated intestinal diseases. In this regard, CLA induced anti-inflammatory property may be of considerable interest in aquaculture. However, this potential anti-inflammatory role of CLA in the intestine of fish has received minimal attention.

Under normal conditions, fish maintain health by defending themselves against microbial pathogens using a repertoire of innate and acquired immune defense mechanisms [20]. In mammals, toll-like receptors (TLRs) are responsible for recognizing MAMPs of microbes to activate intestinal mucosal immunity and modulate the gut microbiota [21]. Meanwhile, Toll-like receptor 4 (TLR4), an important member of the TLR family, recognizes endotoxin or lipopolysaccharides (LPS) from gram-negative bacteria [22]. Moreover, the TLR4/NF-κB signaling is involved in the inflammation induced by LPS in mammals and some fish species [23]. In mammals, the beneficial effects of CLA on immune responses have been observed in animal models and human clinical trials [24]. However, the relationships between dietary CLA and TLR4/NF-κB signaling pathway in fish remain unclear and needs to be further investigated. Fish have developed antioxidant enzymes and antioxidant substances to alleviate adverse effects of oxidative stress [25–27]. Moreover, total antioxidant capacity (T-AOC) reflects the overall cellular endogenous antioxidative capability including both enzymatic and non-enzymatic antioxidants [25]. In addition, fish immune defense system includes non-specific (such as complement 3, C3) and specific immune parameters (such as immunoglobulin M, IgM) [20]. However, little is known about the effect of dietary CLA on total antioxidant capacity and the immune responses. Therefore, this study was performed to investigate the effects of dietary CLA on intestinal morphology, T-AOC and selective immune parameters, and mRNA expressions levels of immune-related gene in grass carp and to provide partial theoretical evidence for the mechanism underlying the improved immune responses of fish by dietary CLA.

2. Materials and methods

2.1. Experimental diets

This study share the same feeding trial with our previous study [28]. Seven isonitrogenous (crude protein: 36 g/100g) and isolipidic (crude lipid: 4.5 g/100g) diets were formulated as shown in Table 1: 0 (control), 0.5 (CLA0.5), 1 (CLA1), 1.5 (CLA1.5), 2 (CLA2), 2.5 (CLA2.5), and 3 (CLA3) g CLA per 100g of feed. The diets were formulated to contain 1% linoleic acid (LA) and 0.5% linolenic acid (LNA), which can satisfy the nutritional requirements of grass carp according to National Research Council (NRC) [29]. CLA was added to the diets as a substitute for coconut oil to maintain a constant lipid level. The diet formulations and chemical compositions are shown in Table 1. Experimental diets were prepared to form pellets (1 mm–3 mm in diameter) by using a laboratory presser. These pellets were oven-dried at 60 °C, and stored at −10 °C before use.

Table 1
Formulation and chemical composition of the experimental diets (g/100g in dry matter).

Ingredients	Diets						
	CLA0	CLA0.5	CLA1	CLA1.5	CLA2	CLA2.5	CLA3
Soybean meal (oil-extracted)[a]	48	48	48	48	48	48	48
Rapeseed meal (oil-extracted)[b]	14	14	14	14	14	14	14
Cottonseed meal (oil-extracted)[c]	11	11	11	11	11	11	11
α-starch	8	8	8	8	8	8	8
Corn starch	7.69	7.69	7.69	7.69	7.69	7.69	7.69
Mineral premix[d]	1	1	1	1	1	1	1
Vitamin premix[e]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C[f]	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Coconut oil[g]	3	2.5	2	1.5	1	0.5	0
CLA[h]	0	0.5	1	1.5	2	2.5	3
Refined linoleic acid[i]	1	1	1	1	1	1	1
Refined linolenic acid[j]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cellulose	5.11	5.11	5.11	5.11	5.11	5.11	5.11
Chemical composition (in dry matter)							
Crude protein	36.02	36.24	36.3	35.98	36.08	36.11	36.25
Crude lipid	4.52	4.48	4.5	4.43	4.6	4.55	4.52
Ash	6.16	6.01	5.98	5.98	5.86	5.86	5.84
Gross energy (kJ/g)	15.2	15.33	15.28	15.22	15.39	15.44	15.48

[a] Soybean meal: Anhui Huayi Agr-livestock Technology Co., Ltd., Hefei, China.
[b] Rapeseed meal: Anhui Huayi Agr-livestock Technology Co., Ltd., Hefei, China.
[c] Cottonseed meal: Anhui Huayi Agr-livestock Technology Co., Ltd., Hefei, China.
[d] Mineral premix (mg kg^{-1} diet): NaCl, 500; $MgSO_4{\cdot}7H_2O$, 7500; $NaH_2PO_4{\cdot}2H_2O$, 12,500; KH_2PO_4, 16,000; $Ca(H_2PO_4){\cdot}2H_2O$,10,000; $FeSO_4$, 1250; $C_6H_{10}CaO_6{\cdot}5H_2O$, 1750; $ZnSO_4{\cdot}7H_2O$,176.5; $MnSO_4{\cdot}4H_2O$, 81; $CuSO_4{\cdot}5H_2O$, 15.5; $CoSO_4{\cdot}6H_2O$, 0.5; KI, 1.5; starch, 225.
[e] Vitamin premix (mg kg^{-1} diet, NRC [29]): Thiamin, 20; riboflavin, 20; pyridoxine, 20; cyanocobalamine, 2; folic acid, 5; $_{calcium}$ pantothenate, 50; inositol, 100; niacin, 100; biotin, 5; starch, 3226; vitamin A (as vitamin A acetate and vitamin A palmitate, 1:1), 110; vitamin D_3 (cholecalciferol), 20; vitamin E (dl-α-tocopherol acetate), 100; vitamin K_3 (as menadione sodium bisulphite), 10.
[f] Vitamin C: L-ascorbyl-2-polyphosphate, 35% ascorbic acid equivalent, Beijing Sunpu Biochemical and Technology Co., Ltd., Beijing, China.
[g] Coconut oil: SUPERCOCO, G.G. Alliance Pte Ltd, Philippines.
[h] $_{QINGDAO}$ AUHAI Biotech. Co. Ltd., Qingdao, China.
[i] Refined linoleic acid (LA): Xi'an Kanglong chemical industry co., Ltd., Xi'an, China; purity > 98%.
[j] Refined linolenic acid (LNA): Xi'an Kanglong chemical industry co., Ltd., Xi'an, China; purity > 98%.

2.2. Fish, experimental conditions, and feeding

Grass carp was ordered from Binzi Hatchery Farm in Hefei, Anhui, China and acclimatized in 10 fiberglass tanks for 20 days prior to the experiment. During acclimation, the fish were fed to satiation with the control diet twice a day. The feeding trial was conducted in a recirculation system containing 28 circular plastic tanks. At the beginning of the trial, the fish (5.08 ± 0.08 g) were subjected to fasting for 24 h. The fish were randomly selected, weighed, and stocked in each tank. Three tanks were randomly assigned to each of the seven dietary groups (40 fish per tank). The feeding trial lasted for 65 days. During the experiment, the photoperiod was set to 12 h light/12 h dark, with light from 08:00 to 20:00. Oxygen was supplied by aeration at a minimum level ranging from 7.0 mg L^{-1} to 7.5 mg L^{-1}. Water temperature was

recorded daily and was maintained at 26.5 ± 0.2 °C throughout the trial. pH was approximately 6.8–7.0. Ammonia-N was measured weekly and retained at 0.05 mg L^{-1} to 0.1 mg L^{-1}. Then, the fish were then hand-fed to apparent satiation twice a day (09:00 and 15:00 h).

2.3. Sampling protocols

At the end of the growth trial, grass carp underwent fasting for 24 h. The fish in each tank were anaesthetized with MS-222. Three fish from each tank were randomly selected, and the intestinal segments (proximal intestine, PI; mid intestine, MI; distal intestine, DI) were removed quickly for morphological analysis. Each segment accounted for approximately 1/3 of the whole intestine. Meanwhile, similar procedure was conducted for the intestinal segments of eight fish in each tank for the measurement of T-AOC and immune parameters and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis.

2.4. Morphological analysis

Intestinal segments (PI, MI, and DI) were fixed in 4% polyoxymethylene overnight [30]. Afterward, the fixed specimens were processed following the conventional histological methods for light microscopy. Tissue blocks were processed into 8 μm-thick sections and stained with hematoxylin and eosin. The tissue sections were examined using an Olympus BX60 with Cellsens standard software. Ten representative sections for each intestinal segment were examined, and the means for all groups were calculated. Fold height, longitudinal section area, enterocyte height, thickness of the lamina propria to the submucosa, and muscularis thickness were measured.

PI segments were prefixed in 2.5% glutaraldehyde solution for 24 h, diced into 1-mm^3 pieces and rinsed thrice with 0.1 M phosphate buffer (pH 7.4) for 15 min. Postfixation was carried out in cold 1% aqueous osmium tetroxide for 1 h. Afterward, the fixed specimens were processed using method described by Dong et al. [31]. Ultrathin sections were examined under a FEI Tecnai (G2 F20 S-TWIN, Eindhoven, The Netherlands) TEM.

2.5. Total antioxidant capacity and immunological parameters analysis

Intestinal segments (PI, MI, and DI) were homogenized in ice-cold, 0.65% physiological saline. The homogenates were centrifuged at 6000 rpm for 15 min at 4 °C, and the supernatant were used for T-AOC, C3, and IgM analysis. All these parameters were performed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Cat. No. A015, Cat. No. E032, and Cat. E025, respectively). In brief, T-AOC was measured using the ferric reducing/antioxidant power (FRAP) assay. It is based on the increase in absorbance at 520 nm of the complex tripiridiltriazine (TPTZ)-Fe (II) in the presence of tissue reducing agents. The absorbance readings are taken during 30 min and the results are expressed as Trolox equivalents. The C3 and IgM contents were measured by ELISA based on the manufacture's instructions.

2.6. qRT-PCR analysis

Total RNA was extracted from the intestinal segments using Trizol. The concentration and quality of the total RNA were assayed using method described by Dong et al. [32]. The primer sequences were designed based on the published sequences from grass carp or by RNA-seq in our laboratory (Table 2; β-actin; interleukin 10, IL-10; transforming growth factor beta 1, TGFβ1; interleukin-1beta, IL-1β; interleukin 8, IL-8; interferon gamma 2, IFNγ2; Tumor necrosis factor alpha, TNFα; TLR4, Myeloid differentiation primary response protein, MyD88; interleukin-1 receptor-associated kinase 1, IRAK-1; TNF receptor-associated factor 6, TRAF6; Transforming growth factor beta-activated kinase 1, TAK1; IκBα, inhibitor of nuclear factor kappa-B kinase subunit alpha, IKKα; inhibitor of nuclear factor kappa-B kinase subunit beta, IKKβ; inhibitor of nuclear factor kappa-B kinase subunit gamma, IKKγ; nuclear factor kappa-light-chain-enhancer of activated B cells p65 subunit, NF-κB p65). qRT-PCR was performed and the gene expression levels were calculated using method described by Dong et al. [32].

2.7. Statistical analysis

Data were presented as mean ± SE. The normality and homogeneity of variances among the groups were tested, and the results were subjected to one-way ANOVA. Duncan's multiple range tests were used to determine the significant ($P < 0.05$) differences between the experimental groups. Principal component analysis (PCA) was performed to examine the correlations between the relative expression levels of anti-inflammatory and pro-inflammatory cytokines, TLR4/NF-κB signaling-related genes, and dietary CLA treatments in the PI, MI, and DI of grass carp. Statistical analyses were conducted on PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Intestinal morphology

In the PI (Table 3, Fig. 1), the fold height, longitudinal section area, and enterocyte height significantly ($P < 0.05$) increased in fish fed with CLA1.5 to CLA3 diets compared to fish fed the control diet. Moreover, the thickness of the lamina propria to the submucosa significantly ($P < 0.05$) increased in fish fed with CLA1.5, CLA2.5, and CLA3 diets, and the muscularis thickness significantly ($P < 0.05$) increased in fish fed with CLA0.5, and CLA1.5 to CLA3 diets. In the MI (Table 3, Fig. 2), the fold and enterocyte heights were significantly ($P < 0.05$) higher in fish fed with CLA1.5 to CLA3 diets compared to fish fed the control diet. By contrast, the muscularis thickness significantly ($P < 0.05$) increased in fish fed with CLA0.5 to CLA3 diets, and a significant ($P < 0.05$) higher longitudinal section area was only observed in fish fed with CLA3 diet. However, the thickness of the lamina propria to the submucosa was unaffected ($P > 0.05$) among the groups. In the DI (Table 3, Fig. 3), the fold height significantly ($P < 0.05$) increased in fish fed with CLA1.5 to CLA3 diets, and the thickness of the lamina propria to the submucosa significantly ($P < 0.05$) increased in fish fed with CLA1.5, CLA2.5, and CLA3 diets. Moreover, a significant ($P < 0.05$) large longitudinal section area was only observed in fish fed with CLA1 diet, and the muscularis thickness significantly ($P < 0.05$) increased in fish fed with CLA0.5, CLA2.5, and CLA3 diets. However, the enterocyte height was unaffected ($P > 0.05$) among the groups.

In the PI of control fish, a well-preserved epithelium consisting of enterocytes was tightly connected to one another, and regularly arranged microvilli were also observed (Fig. 4A). Similarly, normal intestinal epithelium ultrastructure and regular microvilli were observed in the PI in fish fed with CLA0.5 to CLA2 diets (Fig. 4B–E, respectively). However, a slight disorganization of the microvilli was found in the PI in fish fed with CLA2.5 diet (Fig. 4F). Moreover, the PI in fish fed with CLA3 diet showed an extensive mitochondrial vacuolation (Fig. 4G), dilation (Fig. 4H), and loss of cristae (Fig. 4G).

3.2. T-AOC and immunological parameters

The results of the T-AOC, C3, and IgM are shown in Table 4. In the PI, T-AOC was significantly ($P < 0.05$) enhanced in fish fed with CLA1 to CLA2.5 diets compared to fish fed the control diet. C3 contents were significantly ($P < 0.05$) higher in fish fed with CLA1.5 to CLA2.5 diets compared to fish fed the control diet. IgM contents significantly ($P < 0.05$) increased in fish fed with CLA1 to CLA3 diets compared to fish fed the control diet. In the MI, T-AOC, C3 and IgM contents were significantly ($P < 0.05$) enhanced in fish fed with CLA1 to CLA3 diets compared to fish fed the control diets. In the DI, there were no

Table 2
Primer sequences used for the analysis of mRNA expression by qRT-PCR.

Targeted genes	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product length (bp)	GenBank accession no.
β-actin	CGTGACATCAAGGAGAAG	GAGTTGAAGGTGGTCTCAT	120	M25013
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	112	HQ388294
TGFβ1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	104	EU099588
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	120	JQ692172
IL-8	AGGTCTGGGTGTAGATCCACGCTG	TTAGTGTGAAAACTAACATGATCTCT	137	EU047717
IFNγ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	96	FJ766439.1
TNFα	TGTGCCGCCGCTGTCTGCTTCACGCT	GATGAGGAAAGACACCTGGCTGTAGA	291	EU047718
TLR4	GCTGCCCATTATTCCTTCATC	TCCACCTATTCATCTTTGCCTTC	91	RNA-seq (unpublished)
MyD88	CAAATGATGGACTTTACCTACCTG	ACATCTTTCCTTTCGGCTTT	110	FJ843088
IRAK-1	ATACAGGCATCCCAACATAATGG	ACAGCGTAGTCGGTCTTCTAAAG	98	JQ239168.1
TRAF6	TCACTCACTGTCAGATGTC	TGTTGGCTCTTGTGTTCA	114	KC465198.1
TAK1	AGACAGGACAGACACCAAT	CATCTTACAGTGCTGCTCAA	105	KC465197.1
IκBα	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA	122	KJ125069
IKKα	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA	116	KM279718
IKKβ	GTGGCGGTGGATTATTGG	GCACGGGTTGCCAGTTTG	100	KP125491
IKKγ	AGAGGCTCGTCATAGTGG	CTGTGATTGGCTTGCTTT	104	KM079079
NF-κB p65	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	96	KJ526214

significant ($P > 0.05$) differences in T-AOC among dietary groups. C3 contents were significantly ($P < 0.05$) higher in fish fed with CLA1 to CLA3 diets compared to fish fed the control diets. IgM contents significantly ($P < 0.05$) increased in fish fed with CLA1.5 to CLA3 diets compared to fish fed the control diets.

3.3. The mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines in the PI, MI and DI of grass carp fed diets containing graded levels of CLA

In the PI (Fig. 5), the mRNA expression levels of IL-10 and TGFβ1 were significantly ($P < 0.05$) higher in fish fed with CLA1 to CLA2.5 and CLA1.5 to CLA3 diets, respectively, compared to fish fed the control diet. Conversely, the mRNA expression levels of IL-1β and TNFα were significantly ($P < 0.05$) lower in fish fed with CLA1.5 to CLA2.5 diets compared to fish fed the control diet. The mRNA expression levels of IL-

Table 3
Intestinal morphology of grass carp fed diets with different concentrations of CLA for 65 days (mean ± SE, $n = 3$).[a]

Proximal intestine (PI)					
CLA levels	Fold height (μm)	Longitudinal section area (μm^2)	Enterocyte height (μm)	Thickness of the lamina propria to the submucosa (μm)	Muscularis thickness (μm)
CLA0	328.8 ± 18.8^{c}	9346.1 ± 108.6^{cd}	26.89 ± 0.80^{d}	402.7 ± 30.3^{b}	68.33 ± 0.65^{d}
CLA0.5	341.9 ± 72.9^{c}	7756.6 ± 44.8^{d}	29.20 ± 0.74^{d}	449.1 ± 124.7^{b}	105.6 ± 7.64^{bc}
CLA1	340.7 ± 26.5^{c}	7991.4 ± 501.2^{d}	29.28 ± 1.24^{d}	386.6 ± 11.2^{b}	82.98 ± 10.51^{cd}
CLA1.5	869.9 ± 45.6^{ab}	23716.7 ± 1500.6^{a}	38.44 ± 0.38^{c}	974.2 ± 35.3^{a}	121.8 ± 5.36^{ab}
CLA2	680.1 ± 118.0^{b}	13384.9 ± 2530.3^{bc}	43.65 ± 1.01^{b}	523.2 ± 66.2^{b}	123.5 ± 1.89^{ab}
CLA2.5	933.6 ± 57.3^{a}	22908.2 ± 1628.2^{a}	51.53 ± 0.55^{a}	1001.4 ± 39.2^{a}	135.41 ± 17.3^{a}
CLA3	699.1 ± 47.5^{b}	15827.3 ± 929.6^{b}	38.89 ± 0.79^{c}	843.185 ± 69.4^{a}	105.9 ± 7.29^{bc}
Mid intestine (MI)					
CLA levels	Fold height (μm)	Longitudinal section area (μm^2)	Enterocyte height (μm)	Thickness of the lamina propria to the submucosa (μm)	Muscularis thickness (μm)
CLA0	255.1 ± 10.4^{c}	3980.0 ± 65.6^{b}	21.24 ± 0.61^{b}	291.3 ± 18.47	43.14 ± 1.12^{e}
CLA0.5	274.3 ± 20.1^{c}	5966.8 ± 179.7^{b}	22.20 ± 0.51^{b}	434.0 ± 81.81	132.80 ± 0.77^{a}
CLA1	278.4 ± 18.5^{c}	6589.6 ± 344.3^{b}	24.12 ± 1.01^{b}	298.0 ± 27.49	61.26 ± 3.40^{d}
CLA1.5	314.2 ± 25.1^{b}	8679.1 ± 231.9^{b}	32.14 ± 1.12^{a}	393.3 ± 12.98	81.30 ± 9.27^{bc}
CLA2	575.7 ± 45.3^{a}	7245.4 ± 778.8^{b}	33.54 ± 1.21^{a}	269.5 ± 25.49	77.35 ± 5.73^{bcd}
CLA2.5	433.6 ± 37.6^{a}	7921.8 ± 134.2^{b}	38.33 ± 1.17^{a}	349.6 ± 6.40	88.08 ± 2.47^{b}
CLA3	367.1 ± 26.5^{b}	14921.0 ± 4610.7^{a}	30.34 ± 0.89^{b}	589.0 ± 184.58	70.19 ± 5.65^{cd}
Distal intestine (DI)					
CLA levels	Fold height (μm)	Longitudinal section area (μm^2)	Enterocyte height (μm)	Thickness of the lamina propria to the submucosa (μm)	Muscularis thickness (μm)
CLA0	192.7 ± 8.4^{b}	7151.3 ± 716.0^{b}	19.88 ± 0.70	327.5 ± 15.65^{b}	54.72 ± 5.30^{d}
CLA0.5	217.5 ± 20.1^{b}	10121.1 ± 1283.2^{b}	20.20 ± 0.81	221.2 ± 21.0^{c}	79.39 ± 4.29^{b}
CLA1	233.6 ± 28.3^{b}	16496.9 ± 2533.8^{a}	21.14 ± 1.02	218.4 ± 6.97^{c}	48.00 ± 6.62^{d}
CLA1.5	336.4 ± 23.5^{a}	11081.6 ± 767.0^{b}	21.78 ± 0.98	502.6 ± 30.80^{a}	58.98 ± 1.85^{cd}
CLA2	364.7 ± 25.3^{a}	7237.1 ± 714.8^{b}	22.75 ± 1.12	362.5 ± 13.28^{b}	58.36 ± 3.59^{cd}
CLA2.5	333.6 ± 37.6^{a}	8226.2 ± 349.1^{b}	21.44 ± 0.75	489.8 ± 24.43^{a}	73.30 ± 0.50^{bc}
CLA3	312.1 ± 23.4^{a}	7610.6 ± 396.3^{b}	20.89 ± 0.91	534.0 ± 27.13^{a}	133.14 ± 8.51^{a}

[a] Means with the different superscripts within the same column are significantly different at $P < 0.05$.

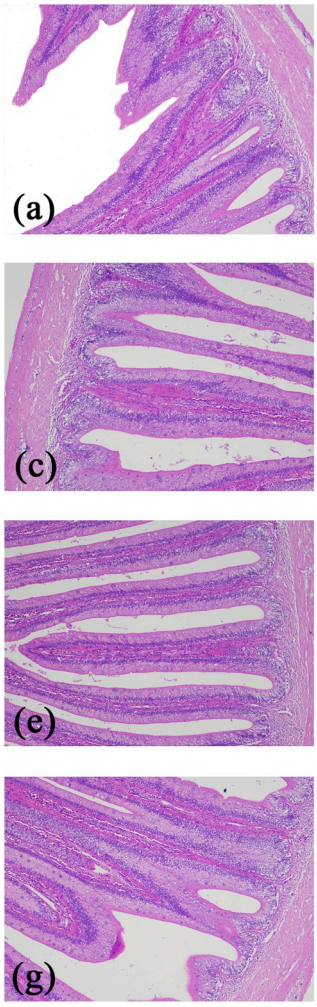

Fig. 1. Representative photographs of the histological morphology sections in the PI of grass carp by H&E staining (magnification, ×100). (a) Section from the control diet; (b) Section from the CLA0.5 diet; (c) Section from the CLA1 diet; (d) Section from the CLA1.5 diet; (e) Section from the CLA2 diet; (f) Section from the CLA2.5 diet; (g) Section from the CLA3 diet.

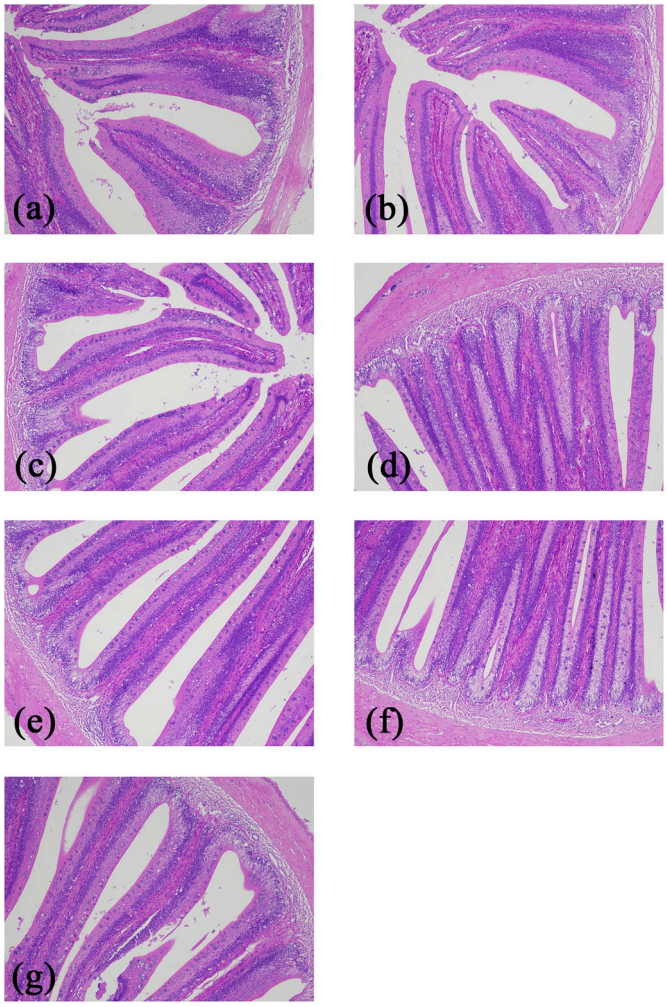

Fig. 2. Representative photographs of the histological morphology sections in the MI of grass carp by H&E staining (magnification, ×100). (a) Section from the control diet; (b) Sections from the CLA0.5 diet; (c) Section from the CLA1 diet; (d) Section from the CLA1.5 diet; (e) Section from the CLA2 diet; (f) Section from the CLA2.5 diet; (g) Section from the CLA3 diet.

8 and IFN-γ2 significantly ($P < 0.05$) decreased in fish fed with CLA1 to CLA3 and CLA1 to CLA2 diets, respectively. In the MI (Fig. 6), the mRNA expression levels of IL-10 and TGFβ1 were significantly ($P < 0.05$) higher in fish fed with CLA1 to CLA2.5 and CLA1 to CLA3 diets, respectively, compared to fish fed the control diet. On the contrary, the mRNA expression levels of IL-1β and IL-8 were significantly ($P < 0.05$) lower in fish fed with CLA1.5 to CLA2.5 diets compared to fish fed the control diet. The mRNA expression levels of IFN-γ2 and TNFα significantly ($P < 0.05$) decreased in fish fed with CLA1 to CLA2 and CLA1.5 to CLA2.5 diets, respectively. In the DI (Fig. 7), the mRNA expression levels of IL-10 and TGFβ1 were significantly ($P < 0.05$) higher in fish fed with CLA1 to CLA2.5 and CLA1 to CLA3 diets, respectively, compared to fish fed the control diet. By contrast, the mRNA expression levels of IL-1β and IL-8 were significantly ($P < 0.05$) lower in fish fed with CLA1.5 to CLA2.5 diets compared to fish fed the control diet. In addition, the mRNA expression levels of IFN-γ2 and TNFα significantly ($P < 0.05$) decreased in fish fed with CLA1.5 to CLA2 and CLA1 to CLA2.5 diets, respectively.

3.4. The mRNA expression levels of TLR4/NF-κB signaling-related genes in the PI, MI and DI of grass carp fed diets containing graded levels of CLA

In the PI (Fig. 8–1), the mRNA expression levels of TLR4, MyD88, TRAF6, and TAK1 were significantly ($P < 0.05$) higher in fish fed with CLA1 to CLA3 diets compared to fish fed the control diet. The mRNA expression levels of IRAK-1, IκBα and IKKγ were significantly ($P < 0.05$) higher in fish fed with CLA0.5 to CLA3 diets, CLA1.5 to CLA2.5 and CLA1.5 to CLA3 diets, respectively, compared to fish fed the control diet. By contrast, the mRNA expression levels of IKKα, IKKβ, and NF-κB p65 significantly (Fig. 8–2; $P < 0.05$) decreased in fish fed with CLA1.5 to CLA3, CLA1 to CLA3, and CLA1 to CLA2.5 diets, respectively. In the MI (Fig. 9–1), the mRNA expression levels of TLR4, IRAK-1, TRAF6, and TAK1 were significantly ($P < 0.05$) higher in fish fed with CLA1 to CLA3 diets compared to fish fed the control diet. In addition, the mRNA expression levels of MyD88 and IκBα were significantly ($P < 0.05$) higher in fish fed with CLA1.5 to CLA3 diets and CLA2 to CLA3 diets, respectively, compared to fish fed the control diet. By contrast, the mRNA expression levels of IKKα, IKKβ, and NF-κB p65 significantly (Fig. 9–2; $P < 0.05$) decreased in fish fed with CLA1 to CLA3, CLA1.5 to CLA3, and CLA1 to CLA2.5 diets, respectively. In the DI (Figs. 10–1), the mRNA expression levels of TLR4 and TAK1 were significantly ($P < 0.05$) higher in fish fed with CLA1 to CLA3 diets compared to fish fed the control diet. In addition, the mRNA expression levels of MyD88 and IκBα were significantly ($P < 0.05$) higher in fish fed with CLA1.5 to CLA2.5 diets compared to fish fed the control diet. Moreover, the mRNA expression levels of IRAK-1 and TRAF6

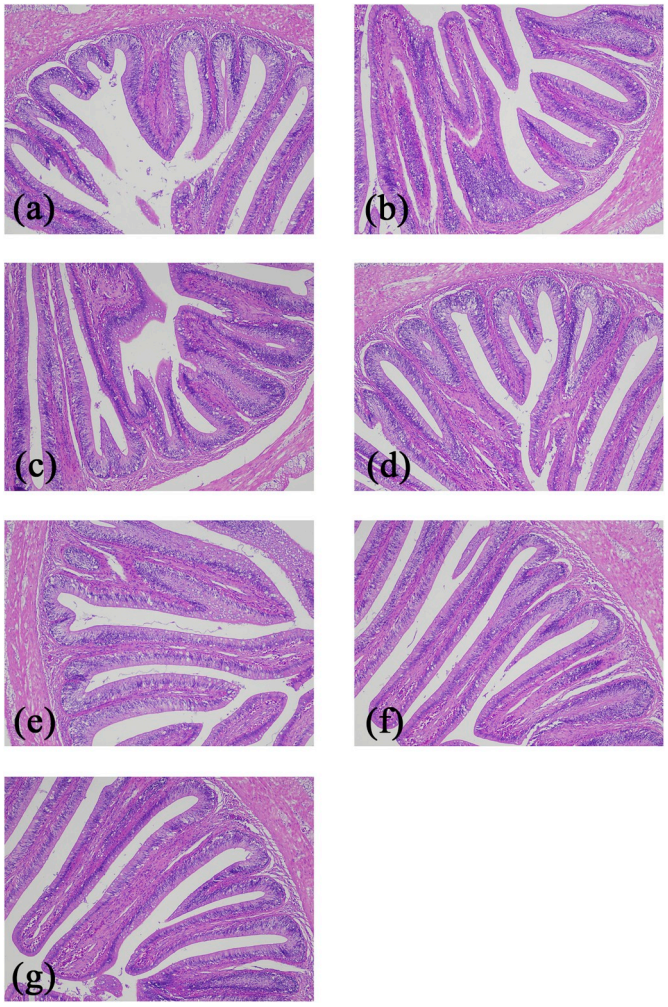

Fig. 3. Representative photographs of the histological morphology sections in the DI of grass carp by H&E staining (magnification, ×100). (a) Section from the control diet; (b) Section from the CLA0.5 diet; (c) Section from the CLA1 diet; (d) Section from the CLA1.5 diet; (e) Section from the CLA2 diet; (f) Section from the CLA2.5 diet; (g) Section from the CLA3 diet.

significantly ($P < 0.05$) increased in fish fed with CLA0.5 to CLA3 diets. By contrast, the mRNA expression levels of IKKα and IKKβ were significantly (Fig. 10–2; $P < 0.05$) lower in fish fed with CLA1.5 to CLA3 diets compared to fish fed the control diet. The mRNA expression level of NF-κB p65 also significantly (Fig. 10–2; $P < 0.05$) decreased in fish fed with CLA1 to CLA2.5 diets.

3.5. Principal component analysis (PCA)

PCA results showed the different correlation patterns between dietary CLA treatments and the mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines, and TLR4/NF-κB-related genes in the PI, MI, and DI of grass carp (Table 5, Fig. 11). In the PI (Fig. 11a), all these indices clustered into three groups: the mRNA expression levels of genes encoding IL-1β, NF-κB p65, IFN-γ2, and TNFα clustered into one group; the mRNA expression levels of TLR4, IRAK-1, TRAF6, MyD88, TAK1, and IKKγ clustered into another group; and the third group comprised the mRNA expression levels of IL-10 and IκBα. In the MI (Fig. 11b), three high aggregations were observed among the mRNA expression levels of IL-8, IL-1β, and TNFα, between the mRNA expression levels of IKKα and IKKβ, and among the genes encoding IRAK1, TRAF6, TLR4, TAK1, and MyD88. In the DI (Fig. 11c), all the indices clustered into three groups: the mRNA expression levels of TLR4, MyD88, IRAK-1, TRAF6, and TAK1 clustered into one group; the mRNA expression levels of genes encoding NF-κB p65, IL-1β, IL-8, and TNFα aggregated into another group; and the third group comprised the mRNA expression levels of IκBα and IL-10.

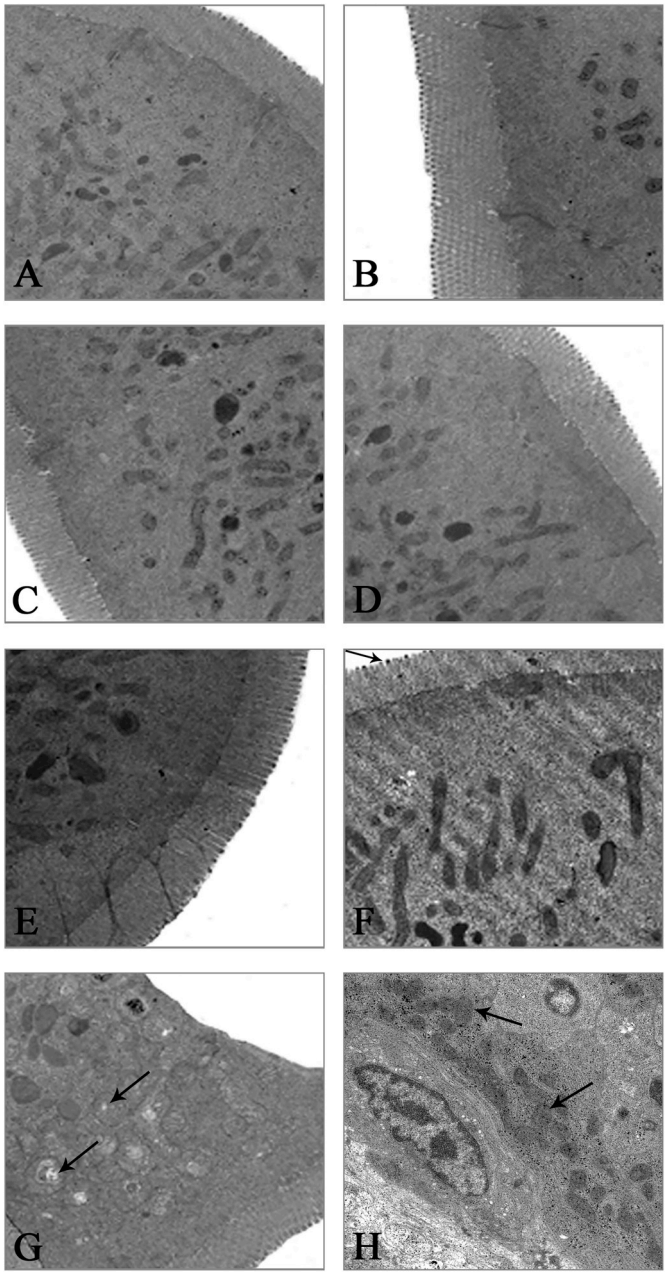

Fig. 4. Cytological morphology in the PI of grass carp fed with different diets (magnification, 3500×). (A) Microvilli and subcellular organelles of the control fish. (B–E) Sections from the fish fed with CLA0.5 to CLA2 diets, respectively. (F) a slight disorganization of the microvilli in fish fed with CLA2.5 diet (black arrow); and (G–H) extensive vacuolation (G, black arrow) and dilation (H, black arrow) in the mitochondrial in fish fed with CLA3 diet.

4. Discussion

4.1. Effect of dietary CLA on intestinal morphology of grass carp

The intestine plays an essential role in the vertebrate physiology as the site of nutrient digestion and absorption [1,33]. Intestinal fold height is an indicator of the absorption ability of aquatic animals [34,35]. Piva et al. [36] reported that short-chain fatty acids derived

Table 4
Total antioxidant capacity and contents of C3 and IgM in the intestine of grass carp fed diets with different concentrations of CLA for 65 days (mean ± SE, $n = 3$)[a].

Proximal intestine (PI)			
CLA levels	T-AOC (U/mg prot)	C3 (g/g prot)	IgM (g/g prot)
CLA0	0.94 ± 0.07^{a}	0.34 ± 0.04^{a}	0.26 ± 0.03^{a}
CLA0.5	0.97 ± 0.07^{ab}	0.39 ± 0.04^{a}	0.23 ± 0.05^{a}
CLA1	1.19 ± 0.08^{b}	0.38 ± 0.05^{a}	0.42 ± 0.06^{b}
CLA1.5	1.33 ± 0.05^{c}	0.55 ± 0.06^{b}	0.63 ± 0.05^{cd}
CLA2	1.43 ± 0.05^{d}	0.76 ± 0.03^{c}	0.76 ± 0.06^{d}
CLA2.5	1.49 ± 0.07^{d}	0.82 ± 0.05^{c}	0.71 ± 0.05^{d}
CLA3	1.05 ± 0.08^{ab}	0.37 ± 0.09^{a}	0.55 ± 0.05^{bc}
Mid intestine (MI)			
CLA levels	T-AOC (U/mg prot)	C3 (g/g prot)	IgM (g/g prot)
CLA0	0.86 ± 0.06^{a}	0.24 ± 0.06^{a}	0.23 ± 0.04^{a}
CLA0.5	0.85 ± 0.03^{a}	0.32 ± 0.04^{a}	0.25 ± 0.07^{a}
CLA1	1.11 ± 0.08^{b}	0.55 ± 0.04^{b}	0.41 ± 0.03^{b}
CLA1.5	1.34 ± 0.07^{c}	0.53 ± 0.06^{b}	0.53 ± 0.07^{bc}
CLA2	1.74 ± 0.05^{d}	0.81 ± 0.06^{c}	0.62 ± 0.05^{cd}
CLA2.5	1.77 ± 0.08^{d}	0.80 ± 0.04^{c}	0.74 ± 0.06^{d}
CLA3	1.33 ± 0.05^{c}	0.62 ± 0.06^{b}	0.63 ± 0.04^{cd}
Distal intestine (DI)			
CLA levels	T-AOC (U/mg prot)	C3 (g/g prot)	IgM (g/g prot)
CLA0	0.74 ± 0.06	0.26 ± 0.03^{a}	0.24 ± 0.09^{a}
CLA0.5	0.61 ± 0.07	0.41 ± 0.06^{ab}	0.23 ± 0.07^{a}
CLA1	0.65 ± 0.10	0.45 ± 0.05^{b}	0.26 ± 0.03^{a}
CLA1.5	0.73 ± 0.05	0.66 ± 0.05^{c}	0.41 ± 0.05^{b}
CLA2	0.68 ± 0.08	0.71 ± 0.09^{c}	0.60 ± 0.04^{cd}
CLA2.5	0.75 ± 0.07	0.69 ± 0.01^{c}	0.73 ± 0.03^{d}
CLA3	0.63 ± 0.05	0.56 ± 0.05^{bc}	0.57 ± 0.04^{c}

[a] Means with the different superscripts within the same column are significantly different at $P < 0.05$.

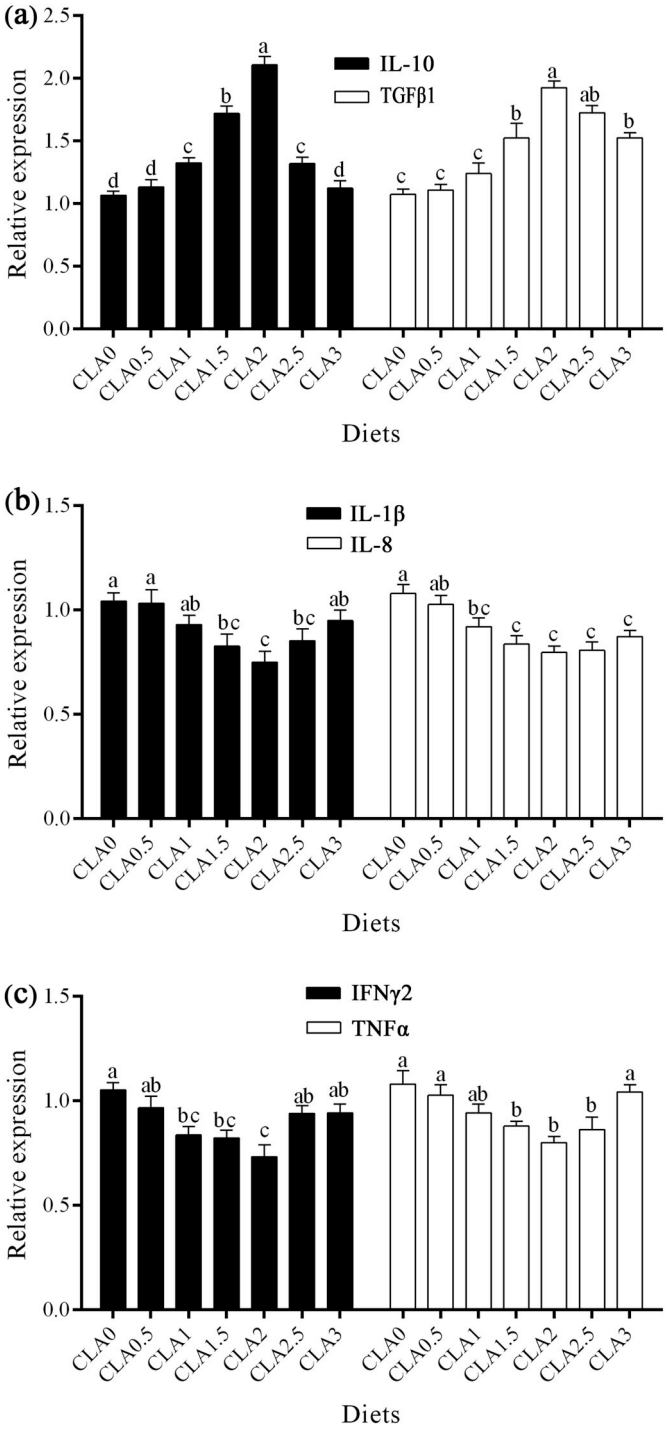

Fig. 5. The mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines in the PI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$).

from a diet supplemented with 10 g/kg tributyrin and 3 g/kg lactitol (alone or in combination) positively affect both cecal crypt depth and ileal villus length in piglets. Likewise, our results revealed that dietary supplementation with 1.5–3 g/100g CLA increased fold height in all the evaluated intestinal segments of grass carp. However, the specific growth rate (SGR) of grass carp decreased from 2.1%/day to 1.95%/day as dietary CLA increased from CLA1.5 to CLA3 [34]. Therefore, these results suggested that dietary supplementation with 1.5–3 g/100g CLA might improve the absorption ability of intestinal segments. However, this improvement could not directly correlate with the growth rate of grass carp. Moreover, the increased fold height in the intestinal segments was possibly a compensatory strategy for the lipid-lowering effect by CLA supplementation. Furthermore, the enhanced enterocyte height was observed in fish fed with CLA1.5 to CLA3 diets in the PI and MI, suggesting that CLA promoted the cell proliferation in the PI and MI of grass carp [37]. These results partly explained that CLA promoted the intestinal fold height of grass carp. The fish fed with CLA1.5 to CLA3 diets showed higher muscularis thickness in the PI and MI than that in fish fed with the control diet. It is possible that CLA can enhance the intestinal muscle development. However, the effect of dietary CLA on intestinal muscle development is poorly understood. Therefore, further study is needed to identify the effect of dietary CLA on intestinal muscle development.

The intestinal epithelium with abundant microvilli is typical of cells involved in absorption and active transport [38]. Therefore, in this study, the normal intestinal epithelium ultrastructure and regular microvilli in the PI in fish fed with CLA0.5 to CLA2 diets suggested that these fish have normal ability in absorption and active transport of nutrients. This finding was consistent with the corresponding specific growth rate in fish fed with CLA0.5 to CLA2 diets [28]. Nevertheless, the microvilli in the PI in fish fed with CLA2.5 diet was characterized by a slight disorganization of the microvilli, suggesting that nutrient

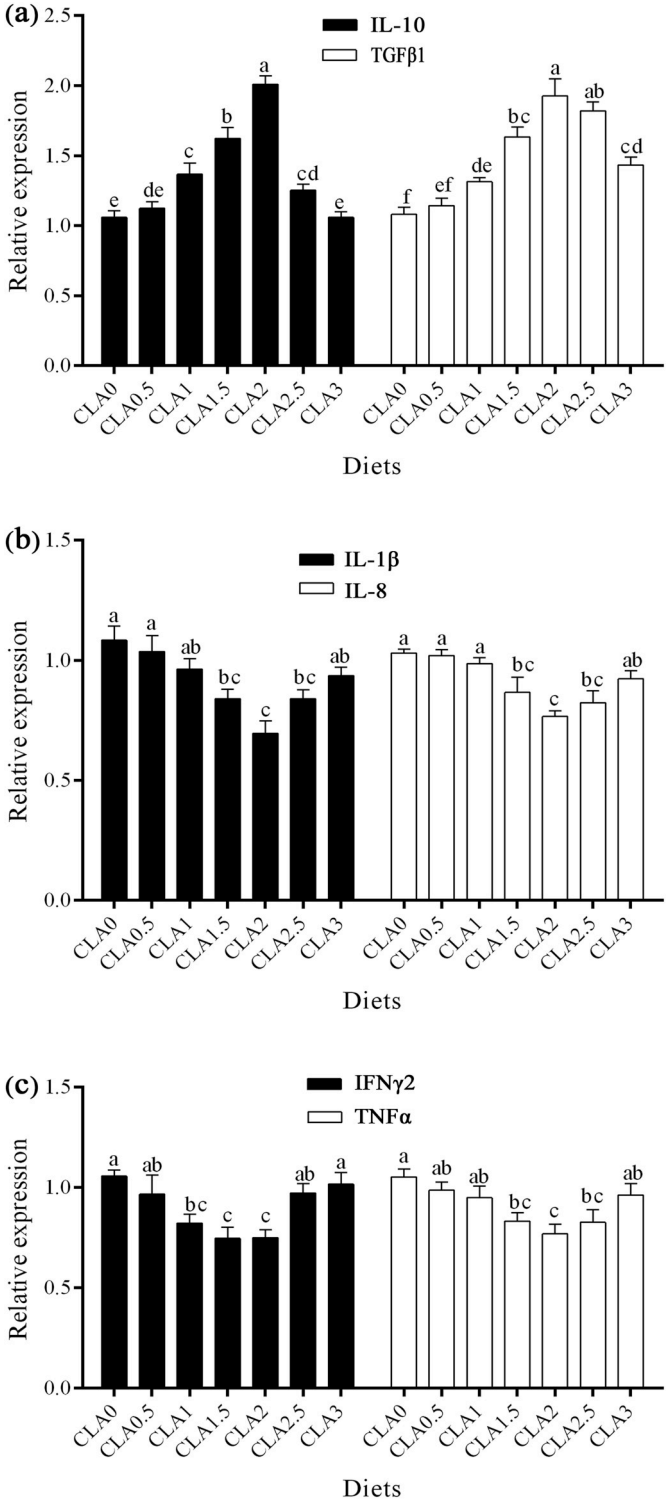

Fig. 6. The mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines in the MI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$).

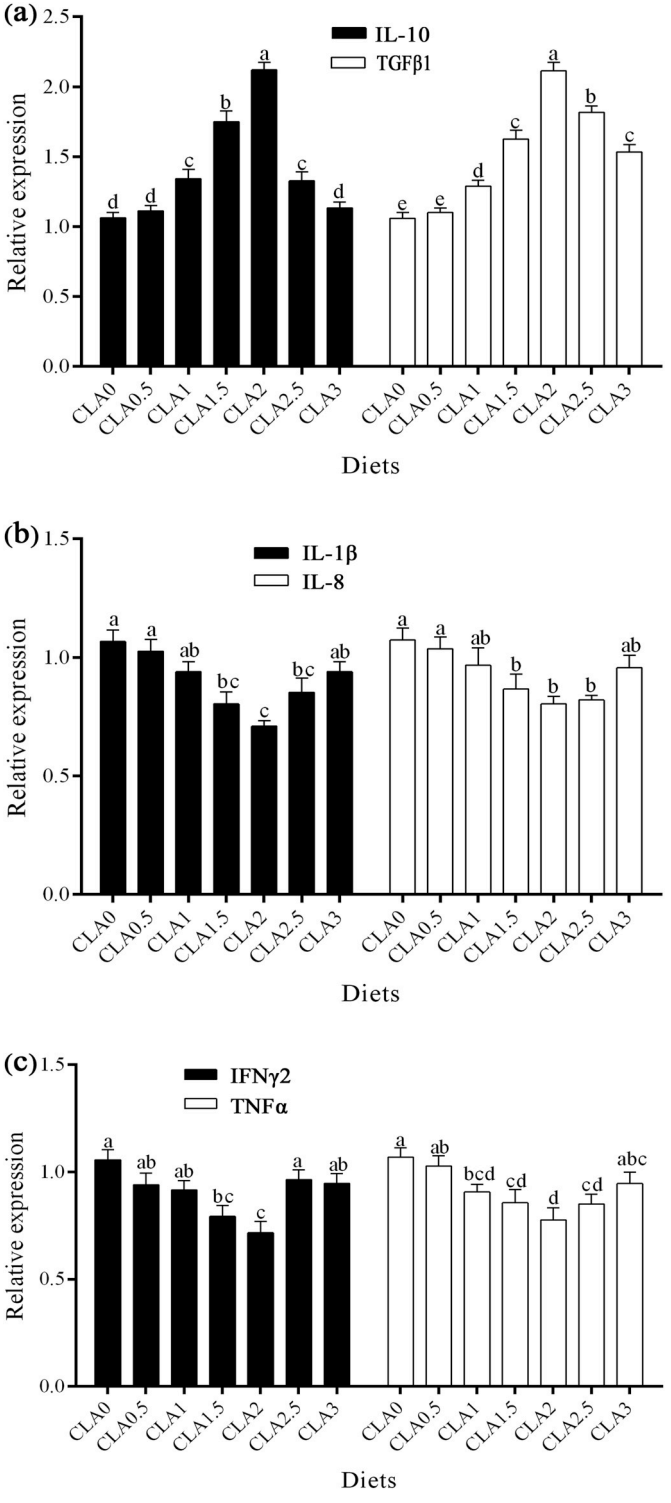

Fig. 7. The mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines in the DI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$).

absorption and active transport in the PI of the fish were affected by supplementation of 2.5% CLA [38]. However, an extensive mitochondrial dilation, vacuolation, and loss of cristae in fish fed with CLA3 diet might be a consequence of the disruptive effects of the hydrogen peroxide–induced inhibitors and disruption of the intestinal epithelium metabolic rate by rendering them energy deficient [38]. Moreover, supplementation of higher level of CLA (3% concentration), a *trans* fatty acid, might have negative effect on intestinal morphology in grass carp [39].

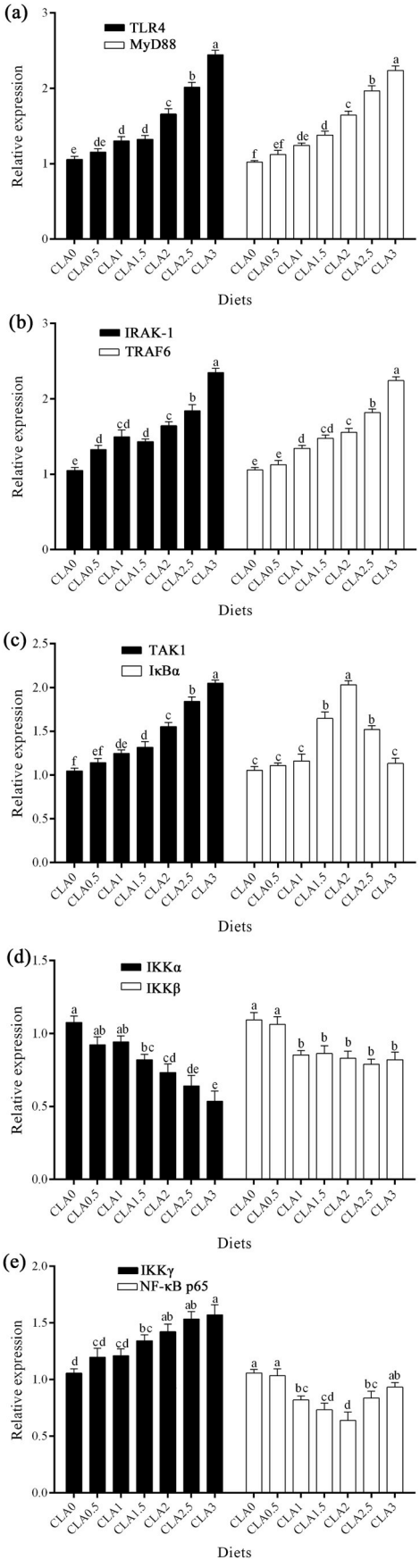

Fig. 8. 8-1 The mRNA expression levels of TLR4/NF-κB signaling-related genes (TLR4, MyD88, IRAK-1, TRAF6, TAK1, IκBα) in the PI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$). **8-2** The mRNA expression levels of TLR4/NF-κB signaling-related genes (IKKα, IKKβ, IKKγ, NF-κB p65) in the PI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$).

4.2. Effect of dietary CLA on T-AOC and immunological parameters in the intestine of grass carp

Martínez-Álvarez et al. [25] suggested that T-AOC reflects the overall cellular endogenous antioxidative capability including both enzymatic and non-enzymatic antioxidants. Therefore, dietary supplementation with CLA increased T-AOC in both PI and MI in fish fed with CLA1.5 to CLA2.5 diets, indicating the enhancement of intestinal antioxidant defenses. Similarly, the T-AOC were significantly enhanced in the liver of yellow perch (*Larmichthys crocea*) when the fish were fed with 0.42% and 0.83% CLA [40]. However, dietary CLA did not affect the T-AOC in DI, suggesting that DI might be less sensitive to dietary CLA than other intestinal segments of grass carp. Magnadóttir et al. [41] reported that C3 plays an important role in recognition and opsonization of bacterial pathogens in fish. Our results showed that dietary CLA enhanced C3 and IgM levels in the intestinal segments of grass carp, suggesting an improvement of non-specific and specific immune responses in grass carp induced by CLA [42].

4.3. Effect of dietary CLA on mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines and TLR4/NF-κB signaling-related genes

Inflammation is a complex physiological response to noxious stimuli and conditions, which result in tissue injury and cell damage [43]. Inflammatory cytokines are classified as pro-inflammatory and anti-inflammatory cytokines [44]. Santana et al. [45] and Fast et al. [46] reported that the up-regulation of pro-inflammatory cytokines, including IL-8, TNF-α, IFN-γ, and IL-1β, and the down regulation of anti-inflammatory cytokines, including IL-10 and TGFβ, initiate inflammatory process. Similarly, we observed that the significantly increased mRNA expression levels of IL-10 and TGFβ1 and significantly decreased mRNA expression levels of IL-1β, IL-8, and TNF-α in fish fed with CLA10 to CLA25 diets in three intestinal segments of grass carp. These results implied that CLA15 to CLA25 diets might reduce inflammation in the PI, MI, and DI of grass carp. Our findings are in accordance with those of Miller et al. [47] and Oleszczuk et al. [48], who proposed that CLA can modify the immune response (modulating TNFα) and prevent immune-induced wasting by influencing the production of soluble factors and inflammatory molecules (cytokines [i.e., interleukin-1, 4, 6, or 8], prostaglandins, or nitric oxides) in mice and domestic animals. Moreover, Zuo et al. [40] found that the mRNA expression levels of genes related to inflammation (cyclo-oxygenase-2, COX-2, and IL-1β) in the liver and kidney significantly decreased as dietary CLA increased in yellow croaker. However, the transcription of TNF-α in the liver and kidney was not significantly different among the dietary treatments in yellow croaker [40]. Therefore, the moderate levels of dietary CLA (1.5g/100g–2.5g/100g CLA) could improve inflammation in the PI, MI, and DI of grass carp by activating the mRNA expression levels of anti-inflammatory cytokines and inhibiting the mRNA expression levels of pro-inflammatory cytokines at least during gene expression in this study.

The TLR4/NF-κB signaling pathways in bony fish could regulate inflammatory cytokines production [23]. Neurath and Pettersson [49]

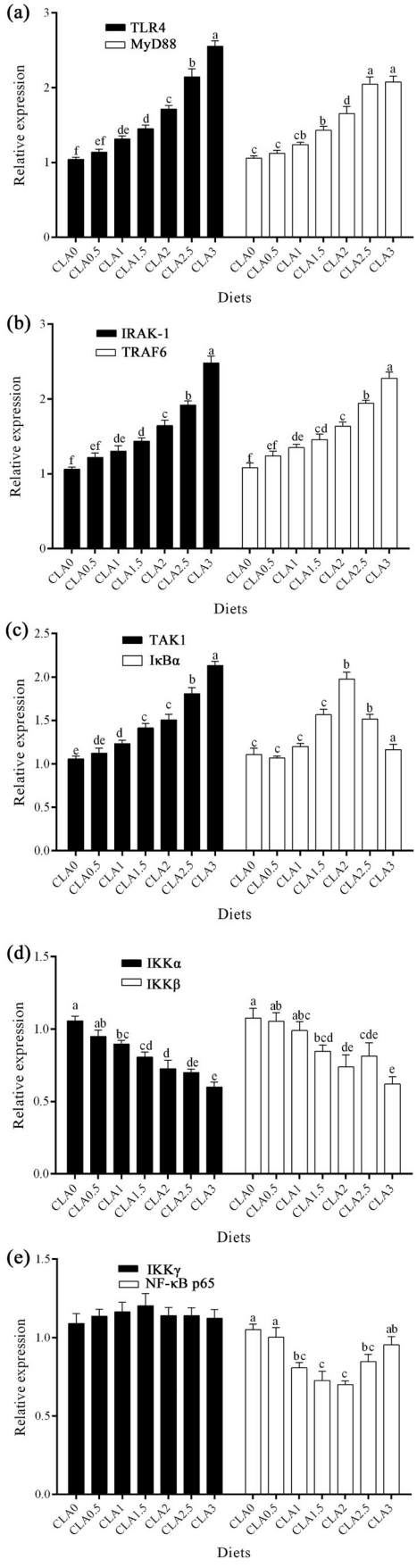

Fig. 9. 9-1 The mRNA expression levels of TLR4/NF-κB signaling-related genes (TLR4, MyD88, IRAK-1, TRAF6, TAK1, IκBα) in the MI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$). **9-2** The mRNA expression levels of TLR4/NF-κB signaling-related genes (IKKα, IKKβ, IKKγ, NF-κB p65) in the MI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$).

and Li et al. [50] reported that up-regulated NF-κB p65 mRNA expression level increase the mRNA expression levels of pro-inflammatory cytokines IL-1β and IL-8 in human cells and the mRNA expression level of TNF-α mRNA level in mouse macrophages. In this study, we found that the mRNA expression levels of TLR4/NF-κB signaling-related genes (TLR4, MyD88, IRAK-1, TRAF6, TAK1, and IKKγ) in the PI, MI, and DI significantly increased in fish fed with CLA1.5 to CLA3 diets. By contrast, the mRNA expression levels of TLR4/NF-κB signaling-related genes (IKKα, IKKβ, and NF-κB p65) in the PI, MI, and DI significantly decreased in fish fed with CLA1.5 to CLA3 diets compared with those in fish fed with the control diet. These results indicated that CLA1.5 to CLA2.5 decreased the mRNA expression levels of pro-inflammatory cytokines IL-1β, and IL-8, and TNF-α, which might be partly attributed to the decreased NF-κB p65 mRNA expression levels in the PI, MI, and DI of grass carp. Previous study suggested that the rapid phosphorylation and degradation of IκBα (a subunit of IκB) could promote NF-κB nuclear translocation, and NF-κB nuclear translocation could initiate the transcription of inflammatory cytokine, including pro-inflammatory cytokines IL-6 and TNF-α in mammals and cells [23,51]. Therefore, IκB could regulate NF-κB nuclear translocation and thus affecting inflammatory cytokine gene transcription. In this study, CLA2 to CLA3 up-regulated IκBα mRNA expression levels in the PI, MI, and DI of grass carp. However, compared to fish fed the control diet, no obvious effects were observed on mRNA expression levels in the pro-inflammatory cytokines, including IL-1β and TNF-α in the PI, MI, and DI in fish fed with CLA2.5 to CLA3 diets. This finding implied that the mRNA expression level of IκBα might be not necessary for the signal dependent activation of nuclear NF-κB [51]. In addition, the increased mRNA expression level of IKKα could stimulate IκBα phosphorylation and degradation, thus increasing NF-κB-dependent mRNA expression level in HeLa cells [52], and increased IKKβ mRNA expression level could induce NF-κB nuclear translocation in NDRG1/Cap43-expressing cells [53]. Therefore, these previous studies suggested that IκB kinases (IKKα, IKKβ and IKKγ) could regulate NF-κB signaling pathway. In this study, compared to the control diet, CLA1.5 to CLA3 significantly decreased IKKα and IKKβ mRNA expression levels in the PI and MI of grass carp, which could be partially responsible for the reduction of NF-κB nuclear translocation. However, no significant effects were observed in IKKγ mRNA expression levels in fish fed with CLA0.5 to CLA3 in the MI and DI of grass carp. This result could be due to the different sensitivity of IKKγ mRNA expression levels in fish fed with dietary CLA in different intestinal segments. Therefore, CLA1.5 to CLA2.5 decreased pro-inflammatory cytokine (IL-1β, IL-8, and TNF-α) mRNA expression levels partly due to down-regulated IKKα and IKKβ mRNA expression levels, which could inhibited IκBα phosphorylation and degradation and reduced NF-κB p65 nuclear translocation in the PI and MI of grass carp.

In this study, PCA analysis indicated that PI, MI, and DI of grass carp showed different correlation patterns between the mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines and TLR4/NF-κB signaling-related genes, which might reflect different regulation strategies in different tissues in response to dietary CLA. In the PI, the mRNA expression levels of genes that encode IL-1β, NF-κB p65, IFN-γ2,

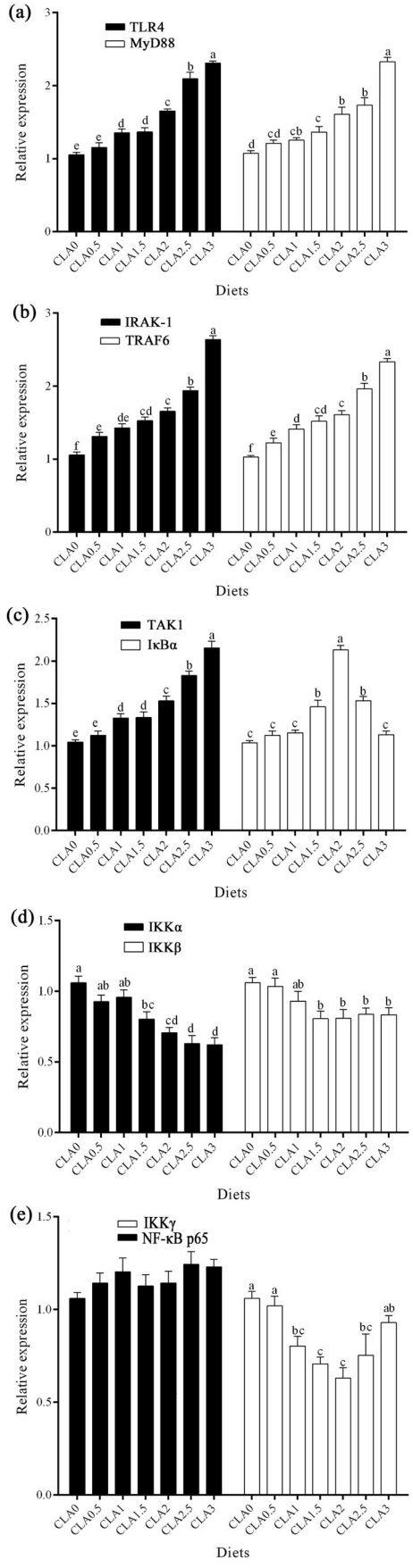

Fig. 10. 10-1 The mRNA expression levels of TLR4/NF-κB signaling-related genes (TLR4, MyD88, IRAK-1, TRAF6, TAK1, IκBα) in the DI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$). **10-2** The mRNA expression levels of TLR4/NF-κB signaling-related genes (IKKα, IKKβ, IKKγ, NF-κB p65) in the DI of grass carp fed diets containing graded levels of CLA (mean ± SE, $n = 3$)*. Means with different superscripts in each parameter are significantly different ($P < 0.05$).

Table 5
Eigenvalues and percentage variance explained of the PCA analysis of dietary CLA treatments and the relative expression levels of anti-inflammatory and pro-inflammatory cytokines and TLR4/NF-κB signaling-related genes in the PI, MI, and DI of grass carp.

Tissues	Initial Eigenvalues			
PI	Component	Total	% of Variance	Cumulative (%)
	PC1	8.82	55.13	55.13
	PC2	6.25	39.04	94.17
MI	Component	Total	% of Variance	Cumulative (%)
	PC1	8.26	51.62	51.62
	PC2	6.50	40.63	92.26
DI	Component	Total	% of Variance	Cumulative (%)
	PC1	8.35	52.20	52.20
	PC2	6.39	39.94	92.14

and TNFα aggregated into one group might suggest that the mRNA expression levels of pro-inflammatory cytokines (IL-1β, IFN-γ2, and TNFα) are highly related to the mRNA expression levels of NF-κB p65. In the MI, the mRNA expression levels of IKKα and IKKβ clustered into one group might suggest that the mRNA expression level of IKKα and IKKβ were mainly responsible for the reduction of NF-κB nuclear translocation. In the DI, the mRNA expression levels of genes, which encoded IL-1β, NF-κB p65, IFN-γ2, and TNFα aggregated into one group, might imply that the mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-8, and TNFα) are highly related to the mRNA expression levels of NF-κB p65. In the PI, MI, and DI, the mRNA expression levels of genes that encoded TLR4/NF-κB-signaling related genes (TLR4, IRAK-1, TRAF6, MyD88, and TAK1) clustered into one group might suggest that MyD88-dependent TLR4-signaling pathway may be involved in the process of inflammatory response after dietary CLA feeding.

In conclusion, our results revealed that dietary supplementation of 1.5%–2.5% CLA improved some selective immune parameters, such as C3 and IgM in three intestinal segments of grass carp. Dietary supplementation of 1.5–3% CLA enhanced the fold and enterocyte heights in the PI and MI. CLA1.5 to CLA2.5 diets increased the mRNA expression levels of anti-inflammatory cytokines (IL-10 and TGFβ1) and significantly reduced the mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-8, and TNF-α) in the PI, MI, and DI. This improved expression of anti-inflammatory cytokines and the inhibited expression of pro-inflammatory cytokines in the intestine of grass carp, might be mediated via TLR4/NF-κB-signaling pathway. Our results suggested that CLA1.5 to CLA2 diets improved intestinal morphology, increased the expression of anti-inflammatory cytokines, and inhibited the expression of pro-inflammatory cytokines in grass carp intestine. Therefore, CLA has the anti-inflammatory therapeutic potential in the intestine of grass carp. This anti-inflammatory potential might be mediated via TLR4/NF-κB-signaling pathway.

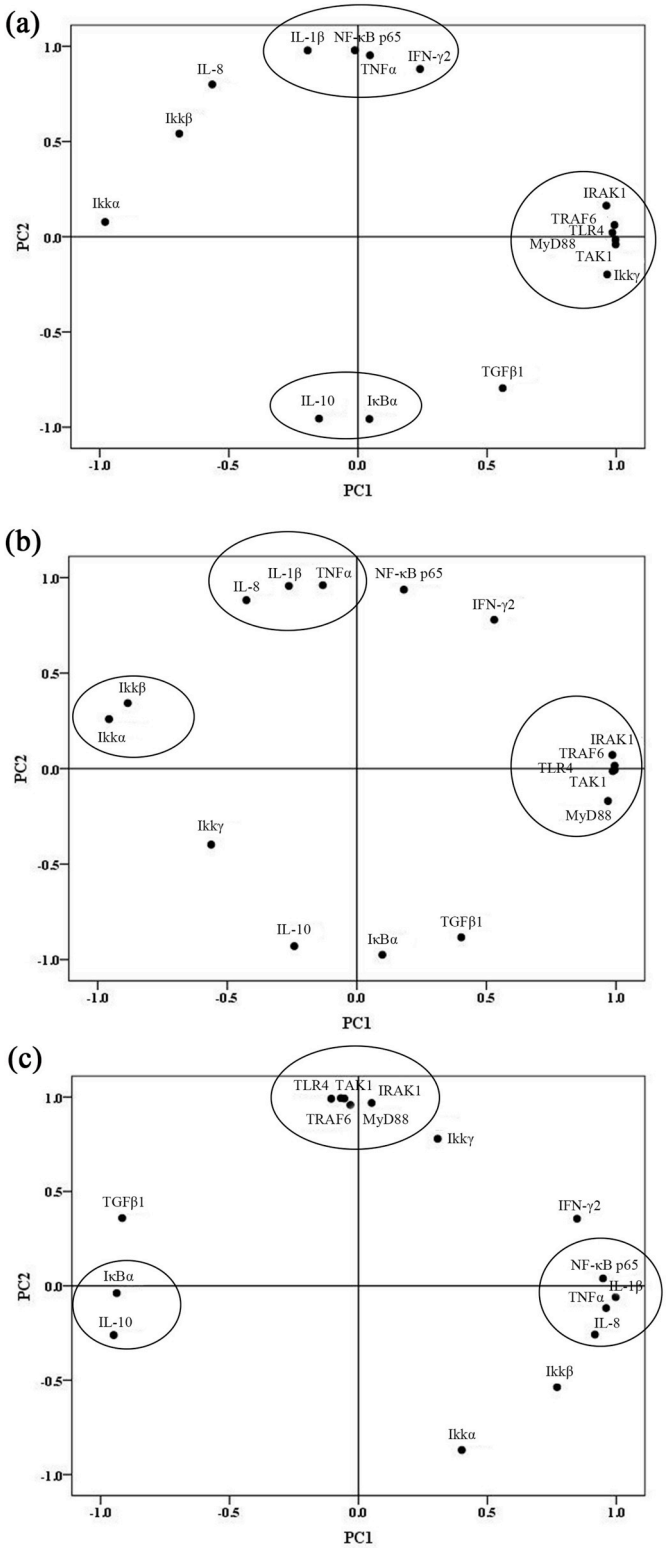

Fig. 11. PCA analysis of the correlations between dietary CLA treatments and the mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines and TLR4/NF-κB signaling-related genes in the PI (a), MI (b), and DI (c) of grass carp.

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